Traditional Chinese Medicine Danshen-Gegen Combination Formula Improves Atherogenic Pathophysiology:

An *in-vitro* and *ex-vivo* Study

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Abstract of thesis entitled:

Traditional Chinese Medicine Danshen-Gegen Combination Formula Improves Atherogenic Pathophysiology: An *in-vitro* and *ex-vivo* Study

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Abstract

Cardiovascular disease (CVD) is the second most common disease in Hong Kong and the leading cause of death in developed countries. Hypertension, smoking, hypercholesterolaemia and diabetes have shown to contribute to this disease. Atherosclerosis is one of the main causes of CVD. The pathology involves oxidation of low density lipoprotein (LDL) by free radicals generated in the body, accumulating at the blood vessel wall. These oxidized LDL are then engulfed by macrophages to form foam cells. Accumulation of foam cells in intima results in plaque development, narrowing of vessel lumens, plaque rupture and blockage of blood flow may occur. Therefore, controlling related atherosclerotic risk factors and oxidation of LDL are the two foci to prevent and relieve CVD.

Danshen and Gegen are two Chinese herbs which have long been using in Chinese tradition to treat CVD. These two herbs are well-known for exerting cardioprotective effects, such as lowering high blood pressure, reducing platelet aggregation and suppressing oxidation stress. Previous studies have shown that the aqueous extract of Danshen-Gegen (Fenge) in 7:3 ratio exhibited potent antioxidative and vasodilative effects which were stronger in potency than that of individual herb. In the present study, further optimization of the formula was undertaken based on these two pharmacological activities. Apart from Fenge, there is another type of Gegen uses in the traditional Chinese medicinal application. Hence another type of Gegen, *Pueraria Lobata* (Yege), was investigated as our alternative herb in the formula. Results showed that 80% ethanol extract of Danshen-Yege (DY 80) exhibited optimal antioxidative effect on AAPH-induced red blood cell hemolysis model and vasodilative effect on U46619-precontracted rat aorta rings.

Results from HPLC analyses revealed the contents of pure compounds in DY 80. Vasodilatory and antioxidative effects of these pure compounds were subsequently investigated using drug concentration in the range comparable to that present in DY 80. Salvianolic acid B (SAB) and daidzein were identified as potent vasodilators, which represent the major active ingredients

contributing to the vasodilation effect of DY 80 extract. At the same time, SAB was found to be the most potent antioxidant which accounted for the majority of DY 80's antioxidative activity. Other compounds such as protocatechualdehyde, tanshinone IIA and puerarin showed less potent antioxidation effect. Furthermore, study of the two compounds purified from Yege, 3'-hydroxypuerarin and 3'-methoxypuerarin, did not show any vasodilatory property.

The mechanisms underlying the vasodilation action of SAB, daidzein and DY 80 were investigated. The results indicated that SAB and DY 80 induced endothelium-independent relaxation, while daidzein caused endothelium-dependent vessel dilation. Further studies using pharmacological blockers demonstrated that SAB and DY 80 mainly acted through the opening of K^+ channels, resulting in hyperpolarization of smooth muscle cells and hence vessel relaxation. The vasodilation effect of daidzein may be mediated by multiple actions. It acted through the nitric oxide (NO) and prostacyclin pathways, and caused the opening of K^+ channels.

The effects of DY 80 and SAB on Cu^{2+}-induced LDL oxidation were examined. The results showed that DY 80 and SAB significantly delayed the

conjugated dienes formation and hence inhibited lipid peroxidation. In the study of oxidized LDL uptake by macrophages, both DY 80 and SAB suppressed the expression of scavenger receptor SR-A on macrophage surface, implicating their role in the inhibition of foam cell formation and hence the prevention of atherosclerosis.

In conclusion, the results indicated that the use of Danshen-Gegen formula for the prevention and/or treatment of atherosclerosis is supported by its pharmacological actions.

中文摘要

心血管病於香港是第二普遍的疾病，而在已發展國家中，心血管病處於頭號殺手的地位。高血壓、吸煙、高膽固醇、糖尿病患者、肥胖、缺乏運動及長期壓力等都是可能引致心血管病的因素。血管粥樣硬化病變是心血管病最主要的成因。其病狀是由於低密度脂蛋白被身體內產生的自由基氧化，積聚於血管壁。 經氧化後的低密度脂蛋白會被巨噬細胞吞噬，形成泡沫細胞。泡沫細胞在血管內膜積聚成爲斑塊，收窄血管內腔，阻礙血液流動。所以，控制低密度脂蛋白氧化的程度及粥樣硬化有關的病變因素成爲防預及減輕心血管病重要的決定因素。

丹參、葛根爲兩種草本藥品長久以來用於中國傳統，以治療心血管病。這兩種草本植物有保護心血管的作用，例如降低高血壓，減少血小板凝聚及壓制氧化壓力等。較早的研究顯示七三比例的丹參-葛根(粉葛)水提物有顯著的抗氧化及舒張血管作用，其功效比單一草本植物顯著。於這次研究中，根據以上兩項作用，方劑的功能獲得進一步提升。這次研究利用葛根的另一種類 - 野葛作材料。結果顯示，丹參-野葛 80% 酒精提取物 (DY 80) 於活體外 AAPH-引發的紅血球溶血實驗中的抗氧化功能以及於 U46619-引發收縮血管的舒張反應都比先前的方劑有明顯的提升。

於純化合物的化學分析實驗中, HPLC 定量測試決定各純化合物於其 DY 80 提取物中的含量。根據其含量, 對這些純化合物的抗氧化及舒張血管作用進行了研究。丹參酚酸 B 及大豆素被確認爲有效引發血管擴張, 以及爲 DY 80 舒張血管作用的最主要成份。同時, 丹參酚酸 B 爲 DY 80 中最有效的抗氧化劑。其他較低效能的抗氧化劑有原兒茶醛, 丹參酮 IIA 及葛根素。此外, 從野葛中提鍊的純化合物, 三-羥基葛根素及三-甲氧基葛根素, 其研究結果顯示沒有顯著的血管舒張效用。

針對 DY 80 提取物及純化合物丹參酚酸 B 及大豆素引發主動脈擴張的機理而進行的研究顯示, DY 80 提取物及丹參酚酸 B 進行非血管內皮組織依賴性的血管擴張作用; 大豆素的血管舒張作用則取決於內皮細胞的完整性, 屬於內皮細胞依賴的擴張作用。進一步的研究指出, DY 80 及丹參酚酸 B 的作用是通過開啓鉀離子(K^+)管道, 引發平滑肌細胞的超極化以達放鬆效果。大豆素則有多樣的作用, 通過其影響於一氧化氮及前列環素的路徑以及開啓鉀離子管道引發超極化的行動引致血管擴張。

DY 80 提取物及純化合物丹參酚酸 B 對於抑制低密度脂蛋白氧化的進一步研究進行於活體外 Cu^{2+}-引發的氧化低密度脂蛋白的實驗模型。結果顯示, DY 80 及丹參酚酸 B 能有效延遲偶合雙烯的形成, 阻止脂肪的過氧化反應。於巨噬細胞攝入氧化低密度脂蛋白的研究中顯示, DY 80 及丹參酚酸

B 能有效抑制 A 類清道夫受體於巨噬細胞表面的表達，顯示其於減少泡沫細胞形成以至於預防粥樣硬化中的角色。

總括而言，研究結果顯示丹參葛根方劑用於預防及治理血管粥樣硬化的運用上是獲得其藥理的支持。

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Abbreviations

4-AP	4-aminopyridine
AAPH	2,2'-azobis-(2-amidinopropane) dihydrochloride
ACE	Angiotensin concerting enzyme
Ach	Acetylcholine chloride
ACN	Acetonitrite
AP-1	Activating Protein-1
$BaCl_2$	Barium chloride
BKca	High-conductance Ca^{2+}-activated K^+ channels
cGMP	Cyclic guanosine monophosphate
CABG	Coronary artery bypass grafting
CAM	Complementary and alternative medicine
COX	Cyclooxygenase
CVD	Cardiovascular disease
DMSO	Dimethyl sulfoxide
DY 80	80% ethanol extract of Danshen-Yege
EDHF	Endothelium-derived hyperpolarizing factor
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial nitric oxide synthase
ESI-MS	Electrospray ionization mass spectrometry
GAP	Good agriculture practice
GC	Guanylate cyclase
GMP	Good manufacture practice
HDL	High density lipoprotein
HMG-CoA	Hydroxy-methylglutaryl coenzyme A
HPLC	High Performance Liquid Chromatography
IC50	50% inhibition concentration
ICAM-1	Intracellular adhesion molecule-1
IKca	Intermediate-conductance channel
Kir	Inwardly rectifying K^+ channels
Kv	Voltage-dependent K^+ channels
LDL	Low density lipoprotein
L-NAME	NG-nitro-L-arginine methyl ester
MCP-1	Monocyte chemotactic protein
NF-κ B	Nuclear factor kappa B
NO	Nitric oxide

NOS	Nitric oxide synthase
NMR	Nuclear magnetic resonance
ODQ	1H-[1,2,4]oxadiazolo-[4,2-alpha]quinoxalin-1-one
oxLDL	Oxidized low density lipoproteins
PBS	Phosphate-buffered saline
PC	Protocatechualdehyde
PGI_2	Prostacyclin
Phe	R(-)-phenylephrine hydrochloride
PTCA	Percutaneous transluminal coronary angioplasty
RBCs	Red blood cells
ROS	Reactive oxygen species
SAB	Salvianolic acid B
SD	Sprague-Dawley
SKca	Small-conductance channel
SMC	Smooth muscle cell
SR-A	Scavenger receptor
TIIA	Tanshinone IIA
TCM	Traditional Chinese Medicine
TEA	Tetraethylammonium chloride
TLC	Thin liquid chromatography
THP-1	Human acute monocytic leukemia cell line
TNF-α	Tumor necrosis factor-alpha
TxA2	Thromboxane A2
U46619	9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$
VCAM-1	Vascular cell Adhesion molecule-1
VSM	Vascular smooth muscle

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Chapter 1

Introduction

1.1 Introduction to Cardiovascular Disease and Atherosclerosis

1.1.1 Cardiovascular Disease

Cardiovascular disease (CVD) is a group of disorders relating to the heart and blood vessels, including hypertension (high blood pressure), coronary heart disease (heart attack), cerebrovascular disease (stroke), peripheral vascular disease, heart failure, rheumatic heart disease, congenital heart disease and cardiomyopathies. CVD kills about 17 million people every year worldwide and therefore contributes to nearly one-third of the global death (World Health Organization, 2004). CVD accounts for 50% of deaths in several developed countries. It has long been thought as an affluent countries' disease due to their fat-rich diet and sedentary lifestyle. However, 78% of all deaths related to CVD occurred in low or middle-income countries, and CVD is affecting more and more people in developing countries. One reason for this is the change of lifestyle in these developing countries to a more Western one under the influence of globalization, accompanied by the adoption of Western diet that is

high in saturated fats. Another reason is the lack of medical devices or dedicated medical support programs for the prevention of CVD, as well as the treatment in acute conditions in these countries.

In Hong Kong, CVD is the second leading cause of death, which account for 23.5% of the total death in year 2001 (Hospital Authority of Hong Kong, 2001), in which heart disease accounts for 14.1% and cerebrovascular disease 9.4%. This percentage is close to the first leading cause of death for malignant neoplasm (34.2%).

Cardiovascular incidents are not necessarily fatal, but often cause impairment of normal lives, requiring long-term medical treatment and enormous healthcare costs. Surgical costs for CVD are tremendously expensive. However, the recurrence of blockage is high (Ross, 1993). More and more factors were being found to increase the chance of getting CVD nowadays. Taking precautions on these factors and adoption of a healthy lifestyle would reduce these risks. Risk factors of CVD include: (i) cigarette smoking; people who quit smoking were found having only half the risk of those who continue to smoke, regardless of how long they smoked before quitting. Quitting also decreases the risk of death after coronary artery bypass surgery or a heart attack.

(ii) diabetes mellitus; glycation of lipoproteins increases the risk of atherosclerosis and glycation occurs when blood glucose is elevated (Vlassara et al., 1985), (iii) family history, (iv) obesity, (v) physical inactivity and unhealthy diet, (vi) increased age and male sex, (vii) folate deficiency, (viii) infections, (ix) psychological stress and (x) low birth weight.

1.1.2 Atherosclerosis

Atherosclerosis is the primary cause of CVD. "Athero" means gruel or paste and "sclerosis" means hardening. It is a process in which deposits of lipids, fibrous elements, cellular waste products, calcium and other substances build up in the inner lining of an artery. Atherosclerosis is a progressive disease which can develop in any artery of the body. However, atherosclerosis usually occurs and affects large and medium-sized arteries. Similar pathology happened in small arteries are termed "arteriolosclerosis". Atherosclerosis affects most frequently the aorta which is the largest blood vessel in the body, the coronary arteries, the carotid, the cerebral arteries and also arteries in the legs and abdomen. In addition, atherosclerosis occurs more frequently at sites of arterial branches.

1.1.2.1 Structure of Arteries

All arteries are comprised of three distinct layers consisting intima, media and adventitia, which are separated by internal elastic lamina and external elastic lamina, but the proportion of these layers varies with the size and function in particular arteries.

Intima – it is the innermost layer of arteries, it is composed of an endothelial layer made up of ellipsoid endothelial cells, overlaying by outlines of connective tissue, smooth muscle cells and macrophages may be found in this layer.

Media – it is the middle layer of arteries and it forms the muscular part of the arteries walls. It is made up of circularly arranged smooth muscle cells, elastic fibers, collagen fibrils and proteoglycans.

Adventitia – it is the outermost layer and is composed of irregularly arranged collagen bundles, scattered fibroblasts, a few elastic fibers and small blood vessels (vasa vasorum) which supply the adventitia and the outer media; macrophages may also be present in this layer. This fibrous layer allows the arteries to stretch but prevent overexpansion of the arteries due to the pressure exerted by blood flow.

1.1.2.2 Pathophysiology of Atherosclerosis

Atherosclerosis is a complex and interrelated biological process which involves the interaction between numerous cellular elements. This process is still not completely understood. However, the "response-to-injury" theory is the most widely accepted one (Libby, 2000; Ross, 1993), with lipoproteins or other risk factors such as hypertension and cigarette smoking as the injurious agents.

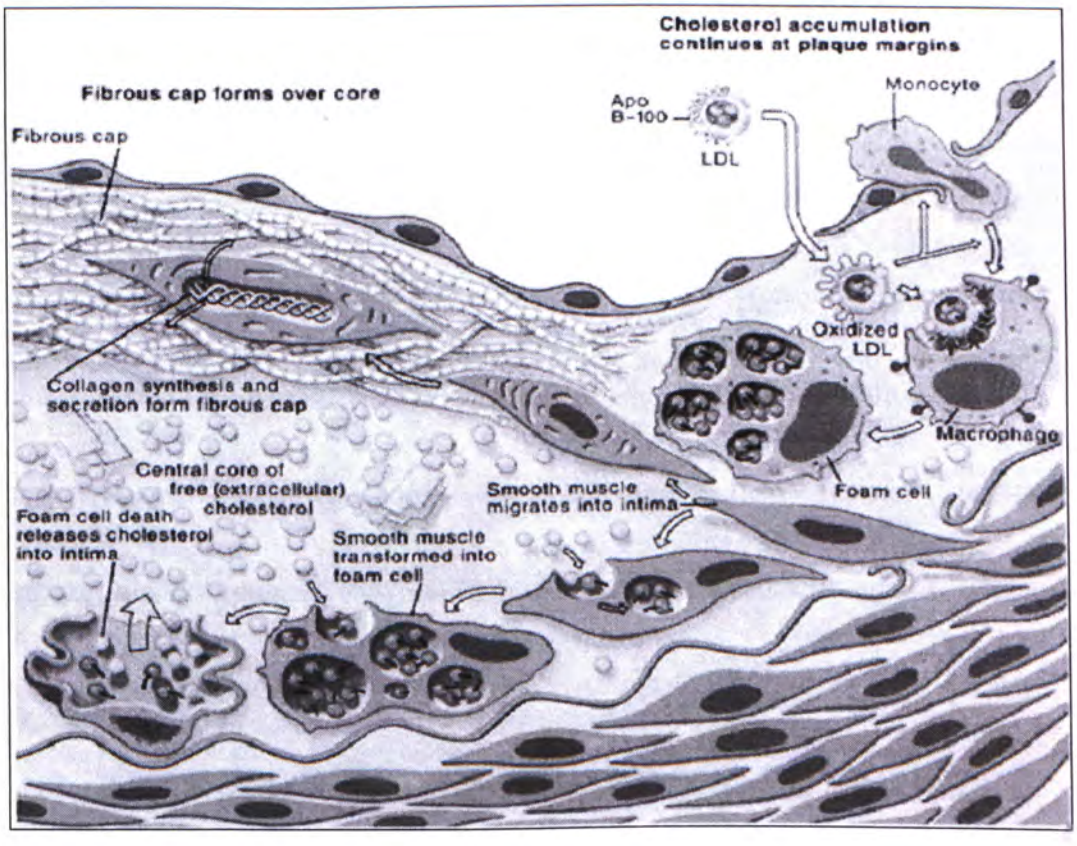


Fig 1.1 Pathophysiology of Atherosclerosis

(Courtesy from Prof. DS Celermajer)

Lesion initiation

The earliest initiating event in atherogenesis is the accumulation of low density lipoprotein (LDL) in the intima. LDL diffuses through the gaps of endothelial cells passively. Diffusion and accumulation increases when the level of circulating LDL is raised and at preferred sites. Cells in the regions of arterial branches, where blood flow is turbulent, show a higher permeability to LDL (Gimbrone, 1999).

Oxidation of Lipoproteins

LDL accumulated in the vessel wall is oxidized by the reactive oxygen species (ROS) or commonly regarded as free radicals in the arteries, to become oxidized lipoproteins (oxLDL). Free radicals are formed as a consequence of many oxidative biochemical reactions in the body. In addition to the environmental free radical sources such as radiation, heavy metals, cigarette smoke, alcohol, etc., oxidized lipoproteins are all chemotactic agents, which can induce the endothelium to express cell-surface adhesive glycoprotein molecules. These glycoproteins then interact with circulating leukocytes, such as monocytes and lymphocytes, enhancing their adhesion and entry into the intima.

Macrophage Migration, Foam Cells and Fibrous Plaque Formation

In response to monocyte chemotactic protein (MCP-1), monocytes transmigrate across the endothelial monolayer into the intima, where they proliferate and differentiate into macrophages and take up the lipoproteins, forming the "foam cells".

Accumulation of foam cells results in yellowish plaque formation in the intima, together with the migration and proliferation of smooth muscle cells from the medial layer, where they secret fibrous elements, resulting in occlusive fibrous plaques which may further increase in size, usually with calcification as well.

Artheriothrombosis

Advanced lesions of plaque can grow sufficiently large to block the blood flow in most atherosclerotic patients. However, the most important clinical complication is an acute rupture or erosion of the lesion, with subsequent occlusion of lumen due to the formation of a thrombus or blood clot, resulting in myocardial infarction or stroke.

In general, arteries affected with atherosclerosis lose their elasticity and reactivity. As the pathological effect of atheroma (plaque) depends largely on the degree of luminal narrowing, lesion or thrombus formation in small arteries

has more vital clinical significance. In addition, atherosclerotic lesions can start at relatively young age (Strong et al., 1999); therefore prevention should be taken as early as possible.

1.1.2.3 Endothelial Dysfunction

Normal endothelium has homeostatic functions include control over thrombosis and platelet, leucocytes interactions, regulation of vascular tone and vascular growth (Celermajer, 1997). The endothelium is also dynamic in secreting powerful vasodilating (e.g. nitric oxide) and vasoconstricting substances (e.g. endothelin-1). Dysfunction of the endothelium is thought to be the key event in the early stage of atherosclerosis, and damage of the endothelium is crucial to the thrombus formation in advanced atherosclerotic lesion. Endothelial injury, either by physical trauma or more subtle cellular damage, promotes atherogenic events by promoting lesion and plaque formation, increasing adherence of monocytes, increasing permeability to monocyte and lipoproteins accumulation in vessel wall, increasing platelet adherence and smooth muscle migration and proliferation. Animal models and human study clearly demonstrated that the endothelial surface is intact in the initial stages of plaque formation. As plaque develops, endothelial denudation,

intima tear and rupture commonly occur (Davies et al., 1988), associated with implications including impaired endothelium-dependent vessel dilation and may induce paradoxical vasoconstriction. Endothelium dysfunction is also associated with direct inactivation of nitric oxide by the excess production of free radicals, which reduce subsequent bioavailability and action of NO (Cai & Harrison, 2000). This situation of endothelial dysfunction was found in patients with hypertension, atherosclerosis and congestive heart failure (Luscher & Vanhoutte, 1986; Wang et al., 1994).

Nitric Oxide (NO)

NO was released by the endothelial cells as a powerful vasodilator in response to stimuli in normal endothelial functions. It was synthesized from L-arginine by nitric oxide synthase (NOS). In addition to regulating vascular tone, NO was demonstrated to have other important roles in the prevention of atherosclerosis. NO can inhibit monocyte adherence to endothelial cells, inhibit smooth muscle cell chemotaxis and proliferation and inhibit platelet adherence and aggregation. NO is also a powerful antioxidant, inhibiting lipid peroxidation in LDL (Rubbo et al., 2000; Hogg & Kalyanaraman, 1998).

However, hypertension was associated with impaired endothelial release of NO. Oxidative stress by free radicals was found to accelerate the breakdown and removal of NO, the reduced bioavailability of NO may contribute to the initiation and progression of atherogensis.

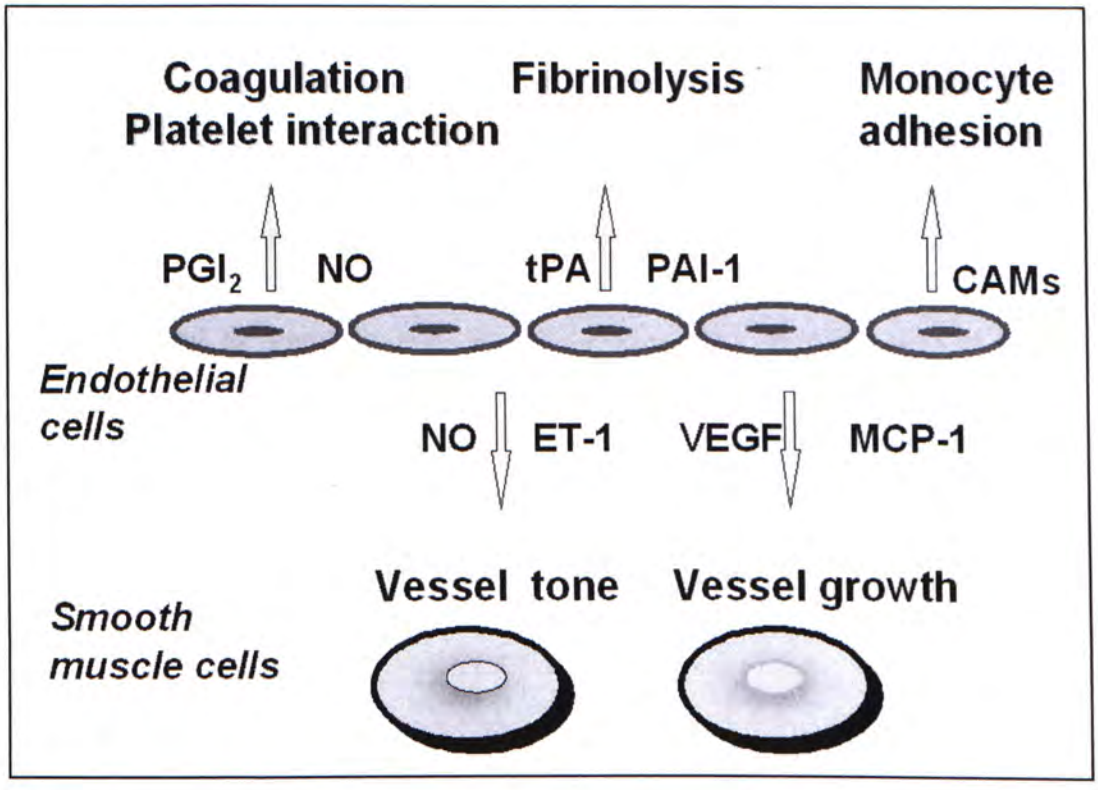


Fig 1.2 Normal endothelial function. *Upward arrows represent factors secreted into lumen. Downward arrows represent factors towards smooth muscle regulating vascular tone and growth. NO was served as a surrogate marker to assess the normal functioning of endothelial layer.* (Courtesy from Prof. D.S. Celermajer)

1.1.3 Current Western Therapies

1.1.3.1 Surgery

For treating cardiovascular disease and mainly the coronary artery disease, percutaneous transluminal coronary angioplasty (PTCA) and coronary artery bypass grafting (CABG) are the two principal procedures utilized nowadays.

Angioplasty

Balloon angioplasty was first introduced in 1977 by Gruntzig. It is a technique used to dilate an area of arterial narrowing with the help of an inflatable balloon or a stent of stainless steel wire mesh. Angioplasty is initially successful in about 95% of patients.

Bypass Surgery

Coronary-bypass surgery was introduced in 1967. Bypass surgery improves the blood flow to the heart with a new route, or "bypass," around a section of clogged or diseased artery. By 1970, a large number of 13,000 Americans has already performed this surgery, among them, 8% died as a result of the surgery. Nowadays, the surgical death rate is reduced to 1-3% owing to technical refinements.

Although both techniques can restore blood flow after surgery, these surgical procedures are invasive, costy and skill-demanding, and the recurrence rate is high. When balloon angioplasty alone is utilized without stenting, the recurrence of the blockage is in the range of 40% in one year. When a stent is used, the recurrence of a blockage is in the range of 15 to 20% in one year. After bypass surgery, about one-third of the patients will develop a recurrence of blockage. These recurrences are due to the inflammation response reacting to trauma either at the site of the original atherosclerotic lesion after angioplasty, or at the site where normal bypass artery or vein is tied to (Anderson, 1992).

1.1.3.2 Western Medications

Western medicines for treating cardiovascular disease have been well-studied and utilized for many decades. There are several types of medications available in the market nowadays. They work through different mechanisms to lower the chance of getting heart attack or stroke.

Cholesterol lowering agents

Bile acid sequestrant and statins are the two groups of medicine which both aim to lower the cholesterol level in circulating blood. Bile acid sequestrant works by enhancing the excretion of bile acids in stool, feedback mechanism would make the liver to convert more cholesterol into bile acids. Statins is also called HMG-CoA reductase inhibitor. HMG-CoA reductase is the enzyme which catalyses the production of endogenous cholesterol.

Anticoagulants and antiplatelet agents

These are medicines that reduce blood clotting in arteries. Antiplatelet agents such as aspirin, act by inhibiting the production of platelet clumping chemicals. Aspirin is the commonest anticoagulant used in controlling acute thrombosis in acute coronary syndrome.

Blockers

Commonly used blockers include α-adrenergic blockers, β-adrenergic blockers and calcium channel blockers. α-adrenergic blockers block the receptors in arteries and smooth muscle cells and relax blood vessels. β-adrenergic blocker blocks the effect of adrenaline on beta receptors and slows down the nerve impulse that travels through the heart and hence the pumping force of the heart. Calcium channel blockers slow down the rate at which calcium passes into the heart muscle and vessel wall. This relaxes the vessels and lowers blood pressure.

Angiotensin System Inhibitor

Angiotensin II is the physiologically active form to activate AT1 receptors to cause vasoconstriction and fluid retention. Angiotensin converting enzyme (ACE) inhibitors work by preventing the formation of this active form from angiotensin I. And angiotensin receptor antagonists block the action of angiotensin II. Both of these actions can increase water loss, lower the blood pressure and reduce fibrosis transformation and muscle hypertrophy.

Diuretics

These drugs are used to help reduce the amount of salt and water in the

body by increasing salt and water excretion in urine, hence lowering the blood pressure.

Adverse Effect

Although western medicines are effective, they usually have side effects. The following list some of the possible side effects of the drugs mentioned.

Cholesterol-lowering agents

These drugs may cause constipation, bloating, heartburn, muscle aches or weakness, abdominal pain or discomfort, diarrhea, and nausea, vomiting, headache and most importantly, hepatic dysfunction.

Anticoagulants and antiplatelet agents

Indigestion is the most common side effect, also gastrointestinal upset including nausea, vomiting, heartburn, epigastric or stomach discomfort, or ulcers and may increase gastrointestinal bleeding.

Blockers

β-adrenergic blockers may cause lethargy, cold hands and feet, nausea, nightmares or vivid dreams, and precipitation of asthmatic attack, fatigue,

dizziness, headache, depression, impotence and decreased sexual drive. α-adrenergic blockers may cause palpitation, dizziness or fainting, especially when accompanied by intake of alcohol, cold hands and feet, temporary impotence, and nightmares, dizziness, nausea and indigestion. Calcium channel blockers can cause headache, facial flush, nausea, tiredness, ankle swelling, dizziness, and rash may occur.

Angiotensin System Inhibitor

Common side effects are dizziness or weakness, loss of appetite, rash, itching, hacking, persistent cough, and swelling.

Diuretics

Although uncommon, lethargy, cramps, rash, or impotence may occur. Some of these adverse effects may be related to potassium depletion.

1.1.4 Traditional Chinese Medicine

1.1.4.1 Long History

Traditional Chinese Medicine (TCM) has a history of over thousands of years. It is powerful because of the fact that it is based on the ancestors' trials and errors and the results of their experience. Types of TCM include acupuncture, remedial massage, herbal medicine and food therapy, Qi Gung and meditation. Among them, Chinese herbal medicine is practicing most widely in Chinese and other Asian populations, and is becoming more and more important in the Western societies. Chinese herbal medicine has been one of the greatest herbal systems of the world, and has an unbroken tradition going back to the 3rd century BC. The earliest recorded Medica, the Sheng Nung Peng Tsao, was written around 2800BC and covered over 300 medicinal substances.

1.1.4.2 As Alternative Medicine

Complementary and alternative medicine (CAM), by definition, is a group of diverse medical and health care systems, practices, and products that are not presently considered to be part of conventional western medicine. Since TCM, especially the herbal medicine is increasingly accepted and used by Westerners in the US and in European countries like Germany, TCM is now commonly regarded as one of the most popular CAM.

It has been recognized that modern western medicines can effectively treat acute or infectious diseases. On the other hand, the strength of traditional Chinese medicine is in treating chronic disorders. Herbal medicine is distinct from single pharmaceutical drug because of the complexity of plant materials. It is more holistic and balanced than medicines based on isolated active ingredients and may be less likely to cause side-effects (Kaufman et al., 1999).

1.1.4.3 Modernization of Chinese Medicine

Modernization of TCM has become a new trend in the field. This involves using scientific methods to investigate the underlining mechanisms of traditional medicines. One important component of this work is to use those instrumentation and methodological tools available in Western Science to investigate the observations and theories made in traditional Chinese medicines.

Part of it is a growing research body which indicates that the action mechanism of traditional herbal medicines could often be explained by modern science. On the other hand, modernization could also be made in the preparation process of the herbal extracts, that is, with the help of scientific research, to optimize the pharmacological value of the tradition medicines. In addition, good agriculture practice (GAP) and good manufacture practice (GMP) are performed to raise the quality and value of tradition medicines. Also formulating traditional Chinese medicine in capsule or by other user-friendly preparations would be a part of this process, which save modern people time in the complicated preparation of Chinese medicines.

1.2 Introduction and Selection of Chinese Medicine

1.2.1 Selection of TCM Formulation from Pharmacopoeia

From the Chinese Pharmacopoeia " Zhong yao fang ji xian dai yan jiu da dian" "中藥方劑現代研究大典" (Huang & Shi, 1996), 28 formulae were stated to exert some sorts of cardiovascular tonic effect. Among these, the simplest formula that has been used to treat human coronary disease is the one that consists of three herbal medicines only. And this formula has been chosen previously by my colleague for investigating the cardiotonic effect. The herbal medicines included in this formula are Danshen, Gegen and Yanhu, and the ratio is in 6:3:1 by weight.

1.2.1.1 Compound Formulation

Herbs are typically prescribed in combinations according to Chinese tradition. Formulae containing more than one single herb were regarded as compound formulation. Compound formulation weights more than single herb because the different components of a formulae can balance each other and they may undergo a mutual synergy which increases efficacy and enhances safety. Therefore, in our study, compound formulation was chosen instead of single herb, for the purpose of obtaining a higher efficacy.

1.2.2 Introduction to the Herbal Medicines

1.2.2.1 Danshen (*Salvia miltiorrhiza*)

Salvia miltiorrhiza, or called Danshen (丹參) belongs to the *Salvia* genus, which is in the *Labiaceae* family containing about 1000 species. Among them, 78 are distributed in China (Wu et al., 1977). Danshen is widely used in the Chinese population. According to the therapeutic theory of Chinese medicine, Danshen is effective in promoting blood circulation, relieving blood stasis, "clearing heat from blood, resolving swelling feeling and tranquilizing the mind". In modern scientific study, Danshen was shown to have multiple pharmacological activities, for instance, anti-platelet aggregation, anti-thrombus formation and improving cardio-cerebral circulation (Chen, 1984). Other cardioprotective effects include antioxidation (Ji et al., 2003), enhancing tissue recovery from anoxia (Cao et al., 2003), anti-atherosclerosis (Wu et al., 1998), anti-hypertension (Kang et al., 2002), hepatoprotection (Wasser et al., 1998) and anti-tumor effect by inducing apoptosis (Liu et al., 2000). This herb is currently and empirically used for the treatment of coronary heart disease, cerebrovascular disease, hepatitis, hepatocirrhisus, chronic renal failure, dysmenorrhea, neurasthenic insomnia (Li, 1998).

1.2.2.2 Gegen (Puerariae thomsonii and Puerariae lobata)

Gegen (葛根), commercially available in markets and traditional Chinese medicine stores, are two different species belong to the same genus *Puerariae* , namely *Puerariae thomsonii Benth.* and *Puerariae lobata (Willd.) Ohwi.* Both species are regarded as Gegen in traditional medicinal use. However, recently, in the 2005 edition of Pharmacopoeia of the People's Republic of China, it has been rewritten that Gegen refers to *Puerariae lobata (Willd.) Ohwi.* only, whereas *Puerariae thomsonii Benth.* is separated out, which is now called Fenge instead.

However, in this study, both species of Gegen were included with reference to the formula previously chosen. Gegen is employed commonly to relieve fever, promote production of body fluid, facilitate eruption, relieve stiffness and pain of the nape (Dictionary of Chinese Traditional Medicine, 1986). Gegen was also shown to lower free and esterified cholesterol in modern research (Lee et al., 2002).

Fenge (*Puerariae thomsonii*)

Puerariae thomsonii Benth., or commonly called Fenge (粉葛), is mostly cultivated in farms and harvested in the fall and winter, it is mainly grown in Guongdong and Guongxi. It contains more starch and is commonly used in preparing household soups. Fewer scientific studies have been done on this herb. Recent research has shown antidiabetic and lipid metabolism- regulating properties (Shen et al., 2006).

Yege (*Puerariae lobata)*

Puerariae lobata (Willd.) Ohwi, or commonly called Yege (野葛), grows wild in nature, at damp places such as in mid of grassland or at road-sides. Yege distributes all over China and is more commonly used in clinical applications. More research studies have been done on this herb, and it is shown to produce hypocholesterolemic (Yan et al., 2006), antiviral, antioxidant (Han et al., 2005) and antitumor effects (Jeon et al., 2005).

1.2.2.3 Yanhu (*Corydalis yanhusuo*) and its Exclusion

Yanhu (延胡), species name *Corydalis yanhusuo*, has been used for improving blood flow and relieving painful conditions (Chevallier, 1996). The root of this herb is analgesic, antiseptic, antispasmodic and sedative. It is used in treatments of disorders including lumbago, dysmenorrhoea, hernia, chest pains, insomnia and traumatic injuries (Yeung, 1985; Duke & Ayensu, 1985).

Exclusion

Yanhu has mainly the analgesic function, which is, alleviating pain without causing loss of consciousness. The inclusion of this herb may lead to the consequence of unnoticeable heart attack, which may delay immediate rescue. Therefore, for precaution and in order to precisely observe the therapeutic effect of the other two herbs in possible clinical trial study, Yanhu has been excluded from the combination herbal formula. The formulation hence only retains the other two herbs, Danshen and Gegen.

1.2.3 Source and Authentication of the Herbal Medicines

The dried roots of the herbal medicines have been undergone authentication before use. Danshen were purchased from local herbal store in Hong Kong which were grown in farm with "Good Agriculture Practice (GAP)" at Sichuan province. GAP farm provides better quality control about the herbs, but not as an absolute guarantee. Dried root of Fenge was purchased from local traditional Chinese medicine store and was grown in Guangxi province in compliance with GAP. Yege was purchased from Guangzhou. The same batches of herbal medicine were used throughout the study to minimize the variance due to different sources. All herbal medicines were authenticated by an expert Dr. Cao Hui (National Engineering Research Center for Modernization of TCM, Zhuhai, Guangdong, China). Morphological identification, thin layer chromatography (TLC) and microscopic structure identification were utilized to confirm that all herbs are genuine medicinal herbs. Voucher numbers for the batches of herbs were as follows.

Herbal Medicine	Voucher no. in storage
Danshen	2005-2793
Fenge	2003-2485
Yege	2005-2792

Table 1.1 Table showing Voucher numbers of Herbal medicines used.

Chapter 2

Optimization of Danshen-Gegen Formula

2.1 Project History

After the elimination of Yanhu, the ratio of the remaining two herbs Danshen to Gegen was changed into 7:3 by Mr. Ken Leung in his thesis entitled "Cardiovascular Tonic Effect of Compound Formula of *Radix Salviae miltiorrhizae* and *Radix Puerariae* 2003", based on the antioxidant property of extracts prepared from a mixture of Danshen and Gegen in different ratios. The results showed that Danshen-Gegen in a ratio of 7:3 has the best antioxidation effect among other extracts in ratios of 2:1 and 1:1. To mention, Fenge and aqueous extract was used in his study. Thereafter, Danshen-Gegen in a ratio of 7:3 was determined and used in the later studies.

2.2 Aims for the Present Study

As mentioned previously, Gegen have two types, *Puerariae lobata* (Yege) and *Puerariae thomsonii* (Fenge). In the previous studies, only the latter with the combination of Danshen was investigated. However, Yege was found to have higher isoflavonoids content than Fenge, which may infer a higher bioactivity (Jiang et al., 2005b). To examine this, the extracts of both Danshen-Yege and Danshen-Fenge combinations were studied for the modification of the formula.

In addition, the two most commonly used methods for processing traditional Chinese medicine are the aqueous extraction and the ethanol extraction methods. Aqueous extracts were obtained using water as the extraction medium. Compounds extracted in this way are mostly water-soluble, hydrophilic substances. However, the water extract yields a large amount of polysaccharides and sugar at the same time, which contributes to much weight of the dried powders. Ethanol extraction is obtained using ethanol as the extraction medium. Ethanol has the tendency to extract non-polar hydrophobic substances and less polysaccharides. A study has shown that the ethanol extract yielded more active ingredients than the aqueous extract (Zhang et al, 1990).

Therefore, in the present study, both ethanol and aqueous extracts of the Danshen-Gegen formulation were investigated for the optimization.

Two pharmacological activities, namely antioxidation and vasodilation, both of which are relevant to the pathophysiology of atherosclerosis, were examined in the present study.

Antioxidation property

It is believed that oxidization of LDL is the precursor in atherogenesis, resulting in inflammatory response and plaque formation (Yla-Herttuala et al., 1989; Carew et al., 1987; Steinberg, 1997). Substances which can deter this process and protect against oxidation are believed to possess antioxidation property.

Vasodilation property

Vasodilation is regarded as a marker for vascular health. Hypertension is found a major cause leading to endothelial dysfunction and atherosclerosis. It was shown to disrupt endothelial integrity and cause damage of the

endothelium (Reidy et al., 1982). Then fibrous plaque formation in the intima blocks the normal blood flow in arteries. Occluded vessels would lead to the deprivation of oxygen to vital organs and result in irreversible damage to the body (Leung et al., 2003; Yee & Schwartz, 1999; Schwartz, 1995). Substances which can relax the blood vessels are said to possess vasodilation property. Potential vasodilators may help relieve hypertension, by restoring the normal functioning of the endothelium, reversing the situation of dysfunction and retarding atherogenesis.

Using antioxidation and vasodilation as monitors, an optimal herbal formula was explored in terms of preparation method and active ingredient content.

2.3 Methods and Materials

2.3.1 Extracts

Due to the differential yield of ingredients extracted by different solvents, aqueous extract, 50% ethanol extract and 90% ethanol extract of the Danshen-Gegen formula were included in the investigation. Aqueous extract is used to retrieve water-soluble substances from raw herbs. 90% ethanol tends to yield organic compounds. And 50% ethanol extract should yield compounds with intermediate polarity. Extracts examined are listed below, all in a Danshen to Gegen ratio of 7:3 in dry weight of the raw herbs.

Extracts	Raw herbs (7:3)	Extraction Solvent
DF Aq	Danshen- Fenge	Distilled water
DF 50	Danshen- Fenge	50% Ethanol
DF 90	Danshen- Fenge	90% Ethanol
DY Aq	Danshen- Yege	Distilled water
DY 50	Danshen- Yege	50% Ethanol
DY 90	Danshen- Yege	90% Ethanol

Table 2.1 Extracts used in formula optimization with different herbal constituents and extraction solvents shown.

2.3.2 Extraction Process

Reflux Boiling

Raw herbs were washed gently with tap water to remove mud and other contaminants. Dried roots were then cut into small pieces. Proper proportions of Danshen and Fenge or Yege were weighed and extracted with the appropriate solvent in a ratio of 1:10 (weight/volume) under reflux for 1 hour. The extract was collected and left until cool. Residues were then subjected to two more subsequent extractions under the same condition for 1 hour and then 0.5 hour. All extracts were pooled and constituted the decoction.

Filtration

The cooled decoction was centrifuged at 7,700 g for 20 minutes and the supernatant was filtered through Walter No.1 filter paper under suction.

Evaporation and Lyophilization

The supernatant was concentrated by rotary evaporator under reduced pressure at 60°C. As for the ethanol extracts, they were evaporated to complete dryness and the extracts were kept in a warmed vacuum desiccator with sodium

hydroxide for 2 days. The concentrated aqueous extract was dried by lyophilization. The dried powder of all extracts was finally collected and kept in the desiccator until use.

Extraction Yield

Extracts	Percentage yield (w/w)
DF Aq	40.5%
DF 50	32.7%
DF 90	7.2%
DY Aq	55.2%
DY 50	16.4%
DY 90	5.5%

Table 2.2 Extraction yield of the six extracts shown in percentage.

2.3.3 *In vitro* Antioxidation Model

AAPH-induced Red Blood Cell Hemolysis Model

2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH) is a water-soluble azo compound which undergoes thermal decomposition to produce carbon-centered free radicals. It has been widely used as a free radical generator in the study of characterization of antioxidants (Niki et al., 1988). The antioxidative capacities of compounds were compared by measuring the inhibition of AAPH-induced hemolysis.

Animals

Male Sprague-Dawley (SD) rats of 320-350g were bred and kept by the Laboratory Animal Services Center of the Chinese University of Hong Kong. All procedures in animal studies have been approved by the Ethical Committee of Animal Studies of the Chinese University of Hong Kong.

Red Blood Cell Suspension Preparation

Rat was anesthetized by ether and blood was collected from the renal vein into heparin-containing tube, then centrifuged at 1500 x g for 10 minutes. Supernatant was discarded. Remaining red blood cells (RBCs) was washed

twice with 0.15M NaCl solution. RBCs were collected by centrifuging at 1000 x g for 10 minutes in the last step. Supernatant was discarded. 5 fold dilution was obtained by adding phosphate-buffered saline (PBS) to the RBCs, resulting in a 20% suspension.

Cell Lysis Reaction

Extracts were weighed and dissolved in PBS or 10% Dimethyl sulfoxide (DMSO). Two-fold serial dilutions with PBS were made to obtain a range of different concentrations of the extracts. Reaction mixtures contain a final concentration of 10% red blood cell suspension, 100mM AAPH and different concentrations of extracts. Control was done by adding PBS or 0.25% DMSO. AAPH was added just before the reaction started. The reaction mixtures were then kept in dark, incubated at 37°C with oscillation for 200 minutes.

Inhibition on Red Cell Lysis

After incubation, each reaction mixture was diluted 20 folds in PBS and distilled water respectively. The diluted mixtures were then centrifuged at 1500 x g for 10 minutes. Supernatant of each reaction tubes was transferred to flat-bottom 96-well plates. Absorbance at 540nm was measured by ELISA plate reader. The percentage of inhibition on the red blood cell hemolysis by

each extract was calculated by the following formula:

$$\text{Inhibition \%} = \left[\frac{DW_{sample} - PBS_{sample}}{DW_{sample}} - \frac{DW_{control} - PBS_{control}}{DW_{control}} \right] \times 100\%$$

The 50% Inhibition Concentration (IC50) was used as an index to compare the antioxidative potency of the test compounds. The lower the IC50 represents a higher antioxidative potency of the compound.

2.3.4 *Ex vivo* Vasodilation Model

Chemicals

R(-)-phenylephrine hydrochloride (Phe), acetylcholine chloride (Ach) and 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$ (U46619) were supplied by Sigma. Krebs solution (118.0 mM NaCl, 4.7 mM KCl, 1.64 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.55 mM D-glucose, 25.0 mM $NaHCO_3$ and 2.5 mM $CaCl_2$) with pH 7.4 was prepared and filtered.

Animals

Male Sprague-Dawley rats of 280-300g were bred and kept by the Laboratory Animal Services Center of the Chinese University of Hong Kong. All procedures in animal studies have been approved by the Ethical Committee of Animal Studies of the Chinese University of Hong Kong.

Aorta Isolation

Rat weighing around 300g was sacrified by cervical dislocation. The aorta was isolated out and soaked in gased cold Krebs solution. After fat and connective tissues were removed, the aorta was cut into 2 mm rings. The aorta rings were then mounted between steel wire hooks and placed into the organ bath. The condition was maintained at 37°C in Krebs solution and gassed with mixture of 95% O_2 and 5% CO_2. The aorta rings were equilibrated for 1 hour, replacing fresh Krebs solution at every 20 minutes interval. And the tension of the rings was gradually increased to 1.5g as the baseline tension.

Aorta Normal Functioning Test

The integrity of the aorta rings was verified before experiments. Aorta rings were first contracted with 0.3 μM Phe, waited for 15 minutes until stabilized,

0.3 μM Ach was then added to relax the aorta rings back to initial baseline tension of 1.5g. Ach mediates an endothelial-dependent relaxation. Aorta rings showing relaxations of 80% or more compared with the Phe-induced contraction tone were used for further drug tests. Those with damaged endothelium were excluded. Aorta rings were then washed and equilibrated again for an hour with successive washing by Krebs solution before drug tests.

Drug Test

U46619 (15 nM) was added to induce contraction of the aorta rings, waited until maximal tension has been reached and sustained. The herbal extracts were then added into the organ baths and the tension was recorded by MacLab data acquisition system through Grass force-displacement transducers FT-03E. Herbal extracts were dissolved in Krebs solution. Dimethyl sulfoxide (DMSO) was used to dissolve extracts which are not soluble in Krebs solution, with a final concentration not more than 0.3% in the organ baths.

2.3.5 Statistical Analysis

All data was shown in mean ± SD. Groups were compared by one way ANOVA with Bonferroni post hoc test analyzed for pairway comparisons and linearity except specified. Statistical significance was inferred at a two sided value of $P<0.05$. GraphPad Prism version 4.00 for window was used for statistical analysis.

2.4 Results

2.4.1 Vasodilation Results

The vasodilation response of the six extracts was compared. In figure 2.1, both 90% ethanol extract of Danshen-Fenge and Danshen-Yege combination showed significant vasodilative effect, which was more potent than their aqueous and 50% ethanol extract counterparts. The latter two did not show significant difference in their vasodilative properties. The difference between the 90% ethanol and aqueous extracts was more obvious in the Danshen-Yege combination.

In figure 2.2, when the combinations of Danshen-Fenge and Danshen-Yege were compared, it was found that the Danshen-Yege extracts generally exhibited a more potent vasodilation response than that of the Danshen-Fenge extracts. The difference in vasodilatory response between the two groups of extracts was significant in their 90% ethanol extracts.

In figure 2.3, when all the six extracts of DF Aq, DF 50, DF 90, DY Aq, DY 50 and DY 90 were compared, it was obvious that 90% ethanol extract of Danshen-Yege combination was significantly more potent in vasodilation response than all the other extracts. Therefore it was the most potent vasodilator among the extracts studied.

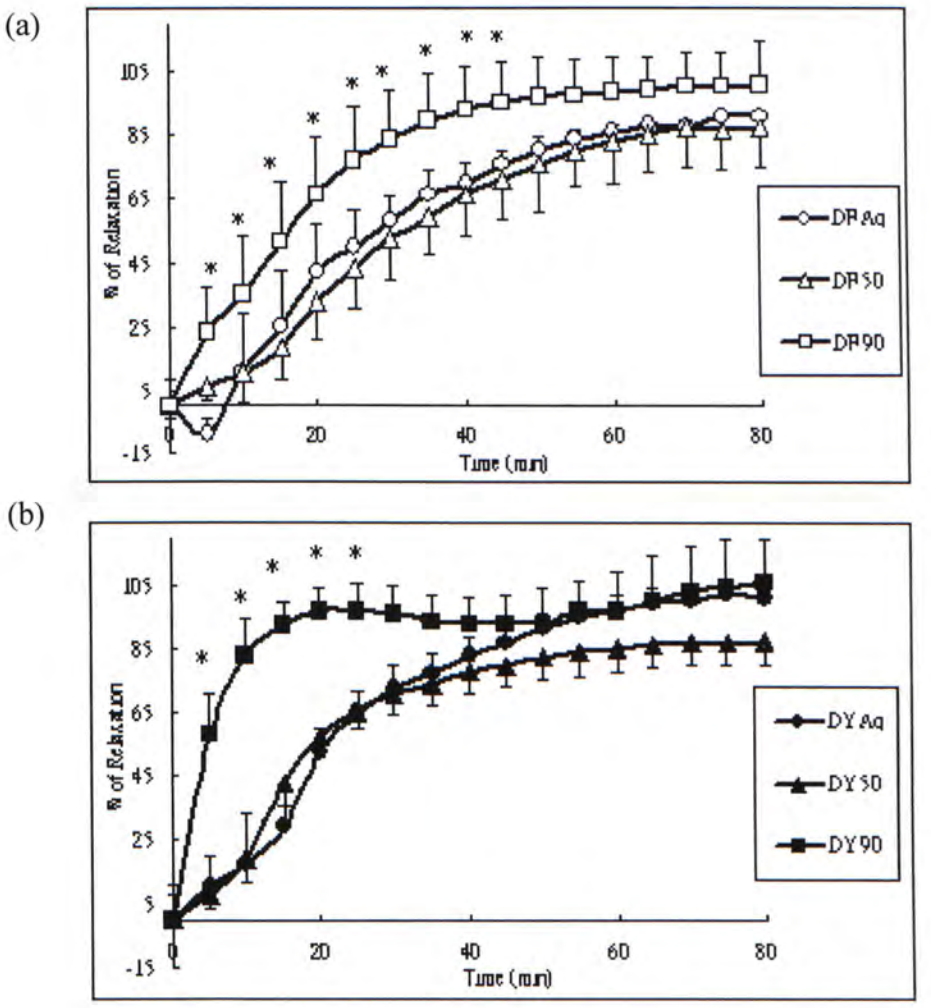


Fig 2.1 Vasodilatory Effect of Aqueous extract versus Ethanol extract of Danshen-Fenge (DF) and Danshen-Yege (DY). *All extracts were administered at Time 0 of 4mg/ml to U46619-precontrated aorta rings. (a) Vasodilation response of DF Aq, DF 50 and DF 90. Effectiveness of dilating effect increased accordingly as DF 50, DF Aq and DF 90. Data were shown as mean ± SD (n=6), * P< 0.05, DF 90 versus DF 50. (b) Vasodilation response of DY Aq, DY 50 and DY 90. Data were shown as mean ± SD (n=6), * P< 0.01, DY 90 versus DY Aq and DY 50. Effectiveness of dilating effect increased accordingly as DY Aq, DY 50 and DY 90.*

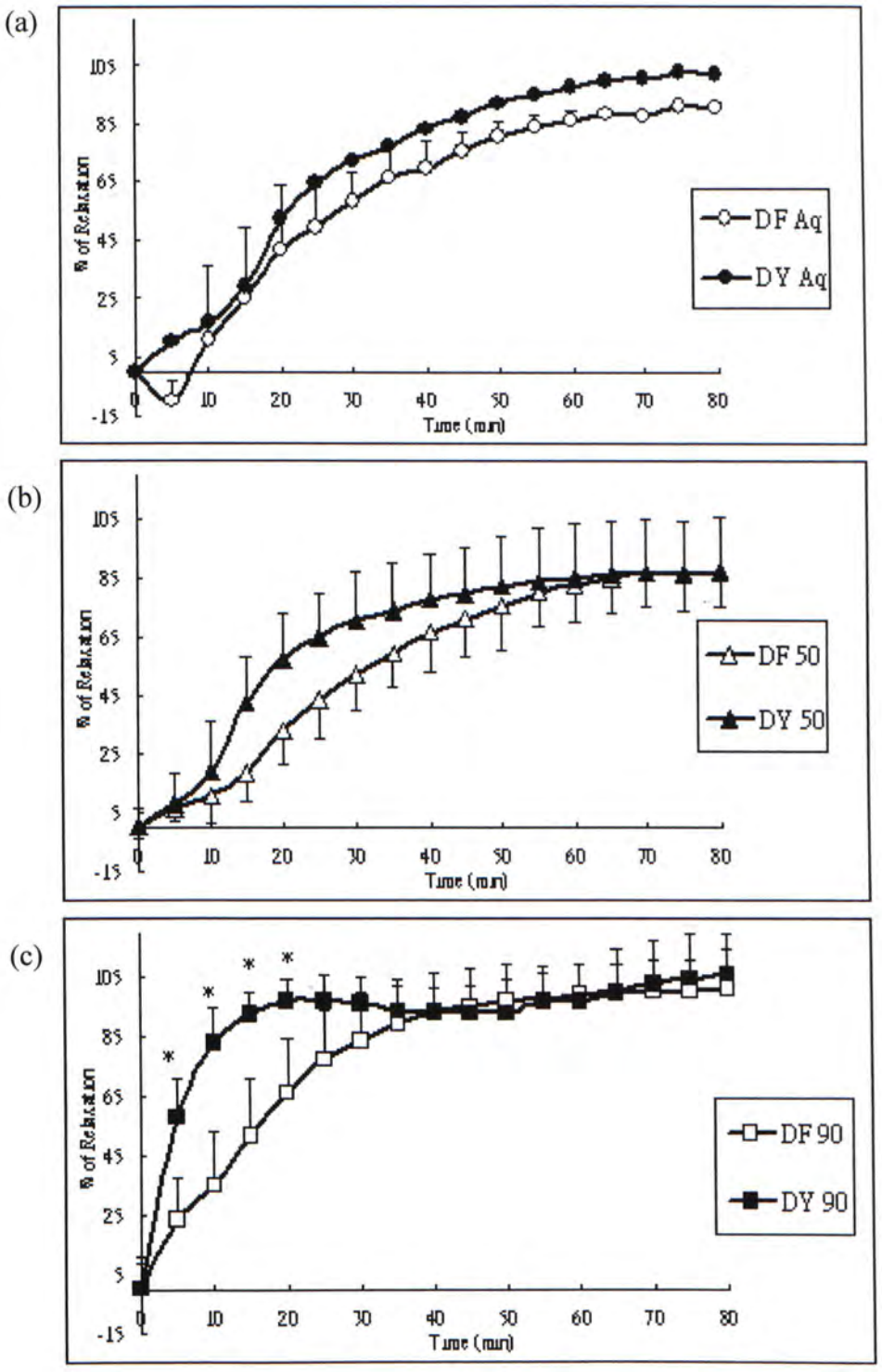
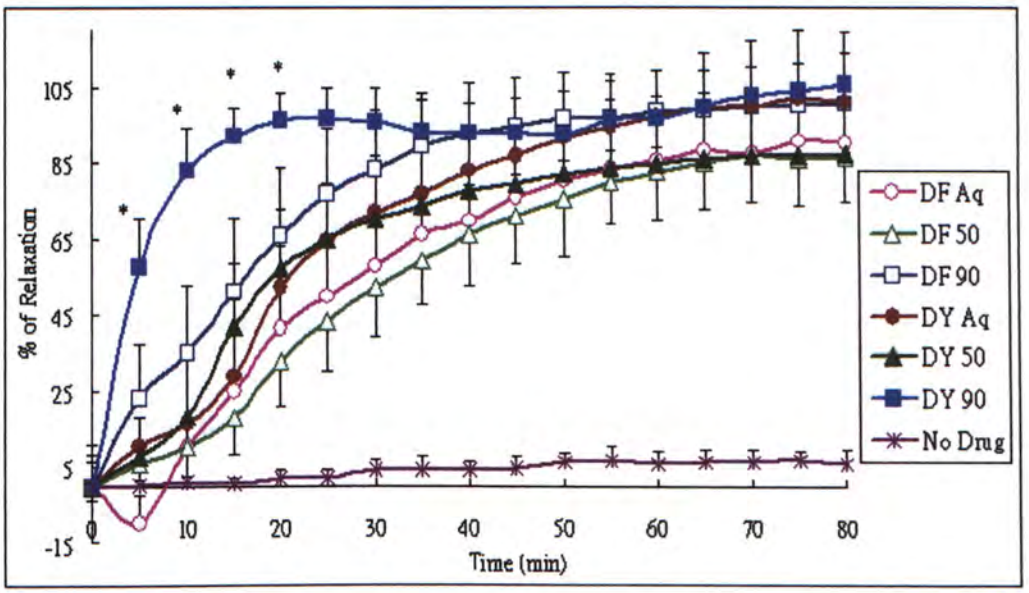


Fig 2.2 Vasodilatory Effect of Danshen-Fenge (DF) versus Danshen-Yege (DY). *(a) Vasodilation response of DF Aq and DY Aq. (b) Vasodilation response of DF 50 and DY 50. (c) Vasodilation response of DF 90 and DY 90. All data were presented in mean ± SD (n=6). Student t-test was used to determine the confident limits between two groups.* * *$P<0.05$*

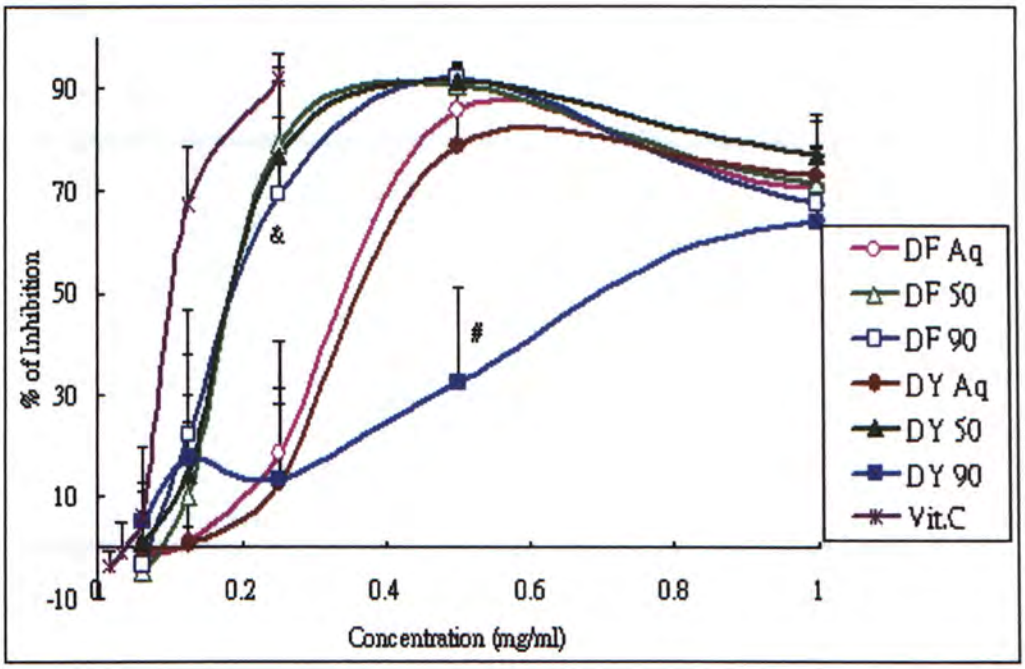


n=6

Fig 2.3 Vasodilation Effect of the herbal extracts on U46619-precontracted aorta rings. *The results were presented in percentage of relaxation induced by drug in the time-course experiment. The concentration of the drugs added were 4mg/ml. Time 0 is defined as the time when drugs were added to the organ baths. Data were shown in mean ± SD (n=6).* * *P<0.05, DY 90 versus other extracts. The effectiveness of the drugs on vasodilative response increases accordingly as DF 50, DF Aq, DY Aq, DY 50, DF 90 and DY 90.*

2.4.2 Antioxidation Results

Antioxidation effects of the six extracts were compared in figure 2.4. 50% ethanol extract of Danshen-Yege, 50% and 90% ethanol extracts of Danshen-Fenge exhibited similar antioxidation activities and were more potent antioxidants than the other extracts, indicated by IC50 value of the extracts in table 2.3. The three extracts, DY 50, DF 50 and DF 90, had the lowest IC50 values (196, 197 and 195 μg/ml respectively) compared with the others. Aqueous extracts of Danshen-Fenge and Danshen-Yege combinations showed a less potent antioxidant effect. However, 90% ethanol extract of Danshen-Yege had a marked reduction in antioxidation activity, with the IC50 value being 782 μg/ml.



n=8

Fig 2.4 Inhibitory Effect of the herbal extracts on AAPH-induced Red Blood Cell Hemolysis. *Results were expressed in the percentage of protective effect of the drugs on AAPH-induced red cells hemolysis. Different dosages of drugs were administered. Vitamin C was used as the positive control. Data were expressed as mean ± SD (n=8).* $^{\&}$ *P<0.05, DF 50, DF 90 and DY 50 versus other extracts.* $^{\#}$ *P<0.05, DY 90 versus other extracts. The antioxidant effect of the extracts increased accordingly as DF 50, DF 90, DY 50 > DF Aq, DY Aq > DY 90.*

Extracts	IC50 (μ g/ml)
DF Aq	322
DF 50	197
DF 90	195
DY Aq	391
DY 50	196
DY 90	782
Ascorbic acid (Vit. C)	105

Table 2.3 Comparison of IC50 values of the herbal extracts and Ascorbic acid. *The protective effect of the extracts on inhibiting red cell hemolysis was compared by IC50 values. IC50 value was obtained by calculating the drug concentration when 50% of inhibition was reached. Unit of IC50 was expressed as μg/ml here. Lower IC50 represents a higher activity of the tested substances. Vitamin C was the positive control. Extracts DY 50, DF 50 and DF 90 have similar IC50 values of 196, 197 and 195 μg/ml respectively. DY 90 has the highest IC50 of 782 μg/ml.*

2.5 Discussion

In the present study, six extracts of DF Aq, DF 50, DF 90, DY Aq, DY 50 and DY 90 were assessed by their vasodilation and antioxidation properties for optimizing the Danshen-Gegen formula, used in preventing atherogenesis and treating cardiovascular disease.

Aqueous and 50% ethanol extracts of both Danshen-Fenge and Danshen-Yege combinations did not show significant difference in the vasodilation activity, implicating that the constituents extracted by water and 50% ethanol were similar in composition and polarities. However, at a high ethanol percentage of 90%, it is possible that more active ingredients for vasodilation were extracted from the raw herbs, as evidenced by a higher bioactivity of the extract. This result is consistent with some previous studies which indicated that ethanol was a more efficient solvent for the extraction of herbs (Zhang et al., 1990; Zhao et al., 2004; Ugochukwu et al., 2003; Savickas et al., 2004). Danshen-Yege combination exhibited a higher potency in vasodilation effect than Danshen-Fenge combination, implying that Yege in general contains more active components than Fenge. A recent study has reported a similar finding that Yege has a higher isoflavonoid content, inferring

a higher bioactivity than that of Fenge (Jiang et al., 2005b). It is also possible that Yege may possess substances which can enhance the solubility or the vasodilatory activity of active components from Danshen. Further study is needed to confirm this. From the present study, the active compounds for vasodilation activity present in Yege were extracted in a greater amount by a higher ethanol concentration. The 90% ethanol extract of Danshen-Yege combination was found to be the most potent in exerting vasodilation effect. In contrast, this extract showed the weakest effect in antioxidative activity. Instead, DY 50, DF 50 and DF 90 possessed more potent antioxidative effects than DY 90. Nevertheless, it is still possible that the ethanol extraction can yield more antioxidant compounds than the aqueous extraction. On the other hand, this different potency in vasodilatory and antioxidant effects (in opposite direction) in different extracts would argue against the pure mass effects of inert polysaccharides extracted out by aqueous and 50% ethanol solvent, and less so in 90% ethanol solvent. This would be reconfirmed in later chapter, after evaluating the concentration of active compounds.

Based on the findings that, (i) ethanol extract possessed more active components; (ii) Yege had a higher vasodilation activity; (iii) DY 90 exhibited the most potent vasodilation response and (iv) DY 50 had the strongest

antioxidant effect, similar to those of DF 50 and DF 90. The optimized formula may somehow be obtained by the extraction of Danshen-Yege mixture using ethanol with percentages ranging from 50 to 90%. Therefore, further studies should compare the 60%, 70% and 80% ethanol extracts of the Danshen-Yege combination.

2.6 Further Modification of the Formula

2.6.1 Extracts

Extracts	Raw Materials (7:3)	Extraction Solvent	Percentage Yield
DY 60	Danshen-Yege	60% Ethanol	28.5%
DY 70	Danshen-Yege	70% Ethanol	23.7%
DY 80	Danshen-Yege	80% Ethanol	16.4%

Table 2.4 Extracts for further optimization with different ethanol concentrations as solvents and percentage yields shown.

2.6.2 Results

Same vasodilation and antioxidation assays used in previous experiments were utilized for the present study. In figure 2.5 and 2.6, vasodilatory and antioxidant activities of 60%, 70% and 80% ethanol extracts of Danshen-Yege combination were compared. Vasodilation responses of the extracts increased when raising the ethanol percentage from 60% to 80%. DY 80 exerted a significantly higher vasodilative effect compared with DY 60. Similarly, the antioxidant effect was enhanced by increasing ethanol percentage during extraction. DY 80 showed a significantly stronger antioxidant effect than those of DY 60 and DY 70 extracts, having the lowest IC 50 of 102 μg/ml (table 2.5).

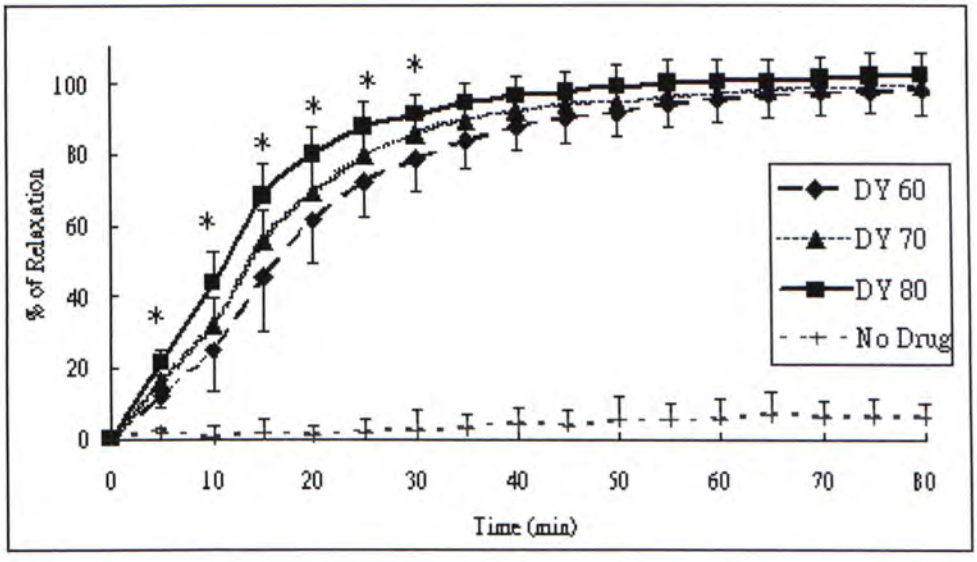
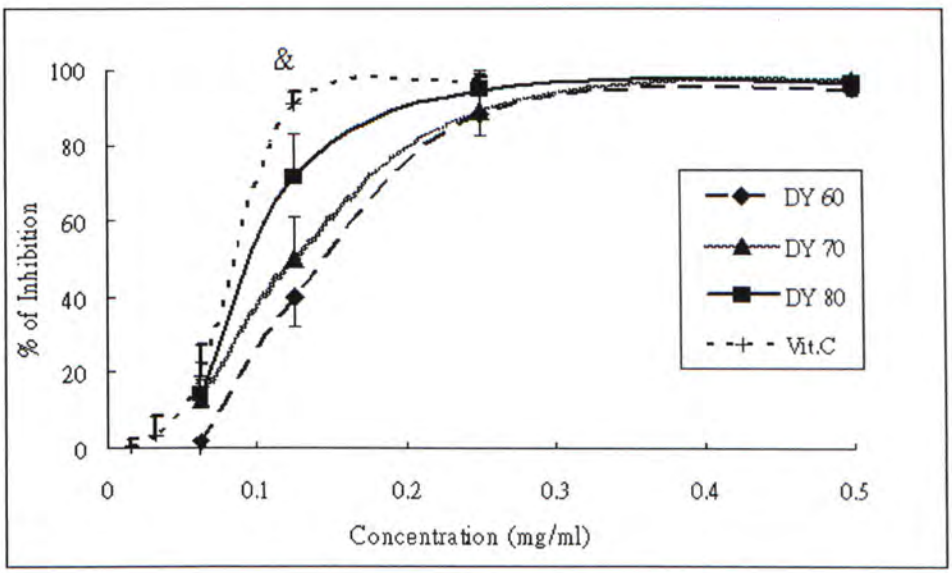


Fig 2.5 Vasodilatory Effect of DY 60, DY 70 and DY 80 on U46619-precontracted aorta rings. *Data were expressed in percentage of relaxation induced by drug added to the pre-contracted aorta ring in a time course experiment. Data were shown as mean ± SD (n=7).* * *P<0.05 DY 80 versus DY 60. Vasodilative response was enhanced by higher ethanol percentage in solvent during extraction. Effectiveness of dilation increased accordingly as DY 60, DY 70 and DY 80.*



n=6

Fig 2.6 Inhibitory Effect of DY 60, DY 70 and DY 80 on AAPH-induced Red Blood Cell Hemolysis. *Results were expressed as the percentage of protective effect of the drugs on AAPH-induced red blood cells hemolysis. Vitamin C was used as a positive control. Data were expressed as mean ± SD (n=6).* $^{\&}$ *$P<0.05$, DY 80 versus DY 60 and DY 70. Inhibitory effect increased with higher ethanol percentages in the solvent during extraction in descending order as DY80 > DY 70 > DY60.*

Extracts	IC50 (μ g/ml)
DY 60	151
DY 70	134
DY 80	102
Ascorbic acid (Vit. C)	93

Table 2.5 Comparison of IC50 values of DY 60, DY 70, DY 80 and Ascorbic acid. *IC50 was obtained by calculating the drug concentration when 50% of inhibition was reached. Unit of IC50 was expressed as μg/ml here. Lower IC50 represents a higher inhibitory activity of the tested substances.*

2.7 Discussion

Vascular contraction is mediated by vascular smooth muscle (VSM) cells. VSM undergoes slow, sustained, tonic contractions. Contraction of VSM can be initiated by mechanical, electrical, and chemical stimuli. Opening of the voltage-gated calcium channels causes the depolarization of the VSM cell membrane and hence contraction. Vasoconstrictors, including norepinephrine, vasopressin, thromboxane A2 and endothelin-1, initiate contraction via increasing the intracellular calcium level.

U46619, used for the precontraction of the aorta rings, is a prostaglandin endoperoxide analog which serves as a thromboxane A2 (TxA2) mimetic that causes vasoconstriction (Burch & Halushka, 1983). Hence the change in the contracted tone could be utilized for the determination of the vasodilation properties of certain substances.

Free radicals are natural by-products generated in many processes within and among cells. They are also created by the exposure to various environmental factors such as tobacco smoke and radiations. Free radicals include hydroxyl radical (·OH), reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$). These molecules are

unstable and highly reactive, damaging cells by chemical chain reactions such as lipid peroxidation, which plays a precursor role in atherogenesis, or formation of DNA adducts that could cause cancer-promoting mutations or cell deaths (Finkel & Holbrook, 2000). The major function of antioxidants is to prevent the damages due to the actions of ROS and free radicals. All cells contain antioxidants that serve this purpose. Dietary antioxidants, therefore, are not the primary antioxidant inside the body, but serve as an external source against the action of free radicals. There are still many questions of how these dietary antioxidants protect cells from oxidation. Some antioxidants were found to preserve, or even recycle, other antioxidants such as vitamin E. Other antioxidants may have far-reaching effects, such as modulating the insulin actions, which is not clearly understood (Nordberg & Arner, 2001).

To the benefits of preventing atherosclerosis, antioxidants can lower the level of cholesterol being oxidized and hence deter the plaque formation and sustain normal blood flow in body. Biomembranes are rich in polyunsaturated fatty acids which are susceptible to the free radical mediated peroxidation. It was shown that low antioxidant status of the erythrocytes membrane increases its susceptibility to hemolysis (Delmas-Beauvieux et al., 1995). AAPH generates free radicals that attack erythrocyte membrane lipids, resulting in lipid peroxidation and eventually hemolysis. This model provides a good way

to evaluate the antioxidative potential of certain substances by examining their inhibitory effect on hemolysis (Niki et al., 1988).

Identification of vasodilators that can restore the vessel dilation function and antioxidants that can reduce oxidative stress, both serve as markers of vascular health, would potentially contribute to the treatment of atherosclerosis. The 80% ethanol extract of Danshen-Yege has been demonstrated to be the most potent in both vasodilation and antioxidation activities. The present study also reconfirmed that the increase in ethanol percentage during extraction could enhance the extract's activity, both in vasodilation and antioxidation. The extract may be therefore used for retarding the progression of atherogenesis and/or preventing the pathogenesis of the disease.

An interesting point to be noted in the modification process of the herbal formula is the solubility of the extract powders, which is also important to be considered, both in facilitating the experimental procedures and for easy consumption as well. In our study, since DY 90 extract contained mostly highly hydrophobic compounds, organic solvent (DMSO) was required for dissolving the extract powders before the administration in experiments. However, the

extracts obtained by 80% or lower ethanol percentage in the extraction process were found soluble in water. This leads to much convenience in the dissolving process and adds value to the optimized formulation for later applications.

Chapter 3

Marker Chemical Contents of Herbal Extracts and their Pharmacological Properties

3.1 HPLC Analysis of Marker Contents

To correlate the pharmacological activities of herbal extracts used in Chapter 2, marker compounds, including protocatechualdehyde, puerarin, daidzin, salvianolic acid B, daidzein and tanshinone IIA, were measured in DY 50, DY 60, DY 70, DY 80 and DY 90 extracts using HPLC method. Moreover, the difference in marker contents of the extracts by raising the percentage of ethanol in the extraction process was compared.

3.1.1 Methods

Calibration curves were constructed using standard marker compounds. Herbal extract samples (10mg/ml) were dissolved in 15% ACN: 85% H_2O and the solutions were filtered and injected into Beckman Ultrasphere ODS, 5μ, 4.6mm x 250mm column via autosampler vials. The mobile phase was A: 0.5% acetic acid and B: ACN. A gradient profile was utilized to obtain the chromatogram. The method used was 0-25min:5-50%B, 25-30min:50-90%B, 30-40min:90%B, 40-45min:90-5%B and finally 45-50min:5%B. Detection wavelengths were set at 254 and 280nm. Samples were measured in triplicate and the content of the respective markers was estimated from the standard calibration curve.

3.1.2 Results

HPLC chromatograms and the concentrations of various markers in the extracts were examined in the present study. Retention times of the six markers were identified in figure 3.1, which were used for identifying the compounds in HPLC chromatograms of the extracts (figure 3.2 - 3.6). It was shown in the HPLC chromatograms that the contents of the marker compounds (puerarin, daidzin, daidzein and tanshinone IIA) generally increased as the ethanol percentage was raised in the extraction process.

3.1.2.1 HPLC Chromatograms

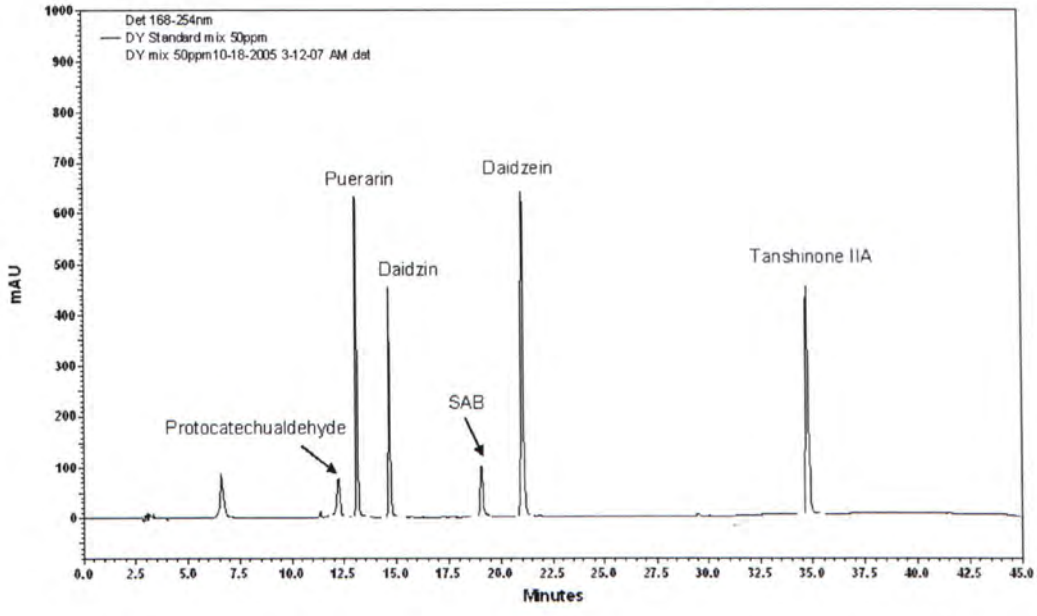


Fig. 3.1 HPLC Chromatogram of a mixure containing six marker compounds. *A mixture of markers with concentrations at 50ppm was shown in this figure. The peaks and retention times of the six markers were identified. Retention times for protocatechualdehyde, puerarin, daidzin, salvianolic acid B, daidzein and tanshinone IIA were identified approximately as 12.2, 13.2, 14.5, 19.0, 21.0 and 35.2 min, respectively.*

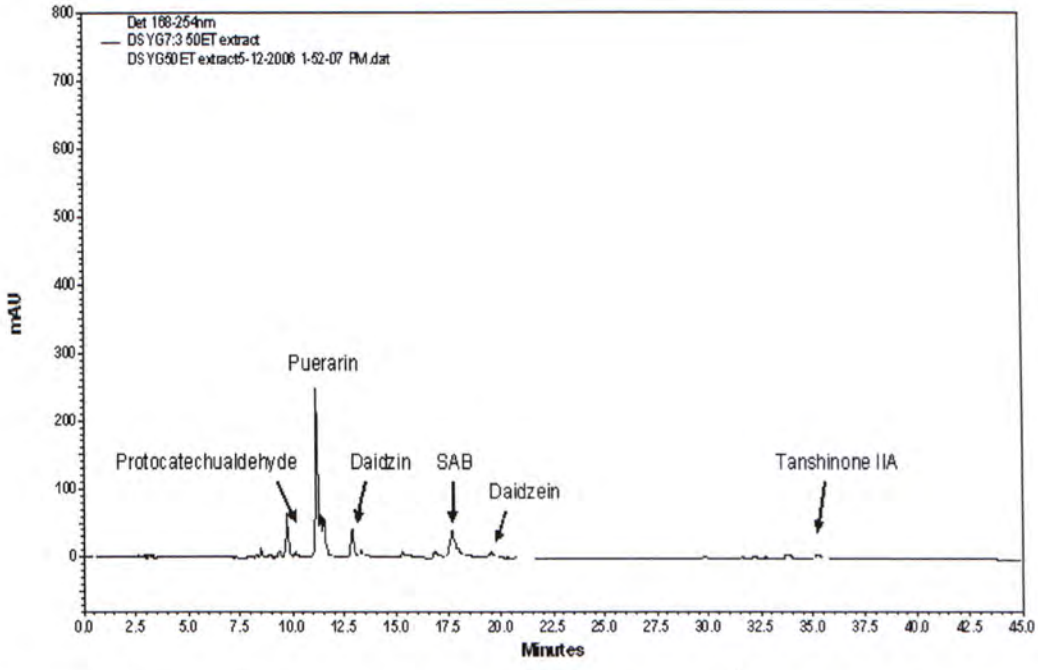


Fig. 3.2 HPLC Chromatogram of DY 50 Extract. *The peaks and retention times of the six markers were shown. The detection was monitored at 254nm. One out of three triplicates was shown here.*

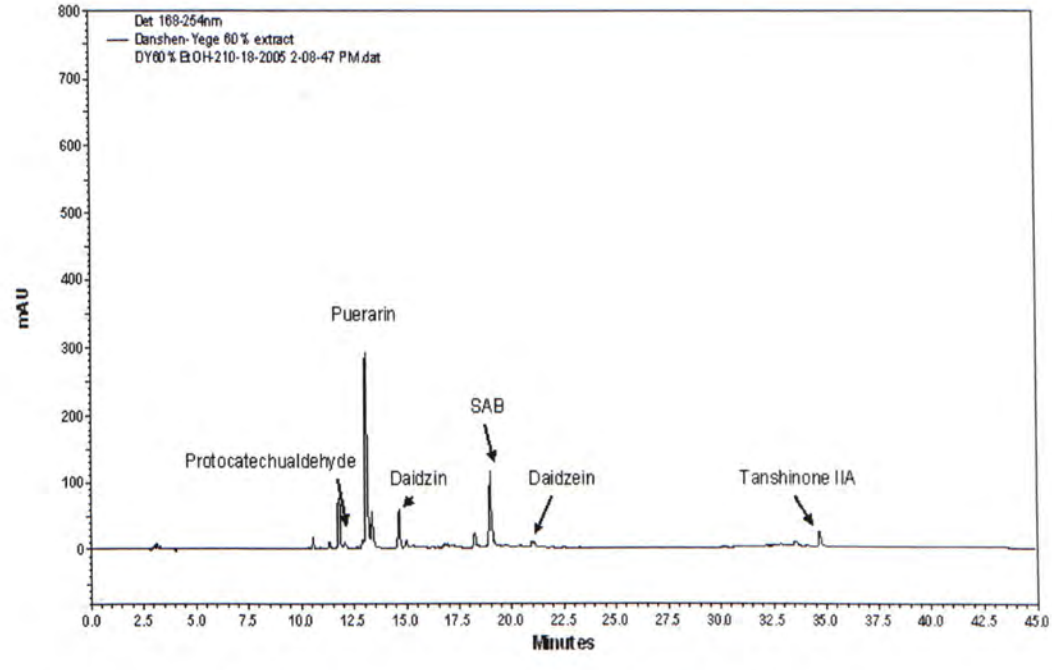


Fig. 3.3 HPLC Chromatogram of DY 60 Extract. *The peaks and retention times of the six markers were shown. The detection was monitored at 254nm. One out of three triplicates was shown here.*

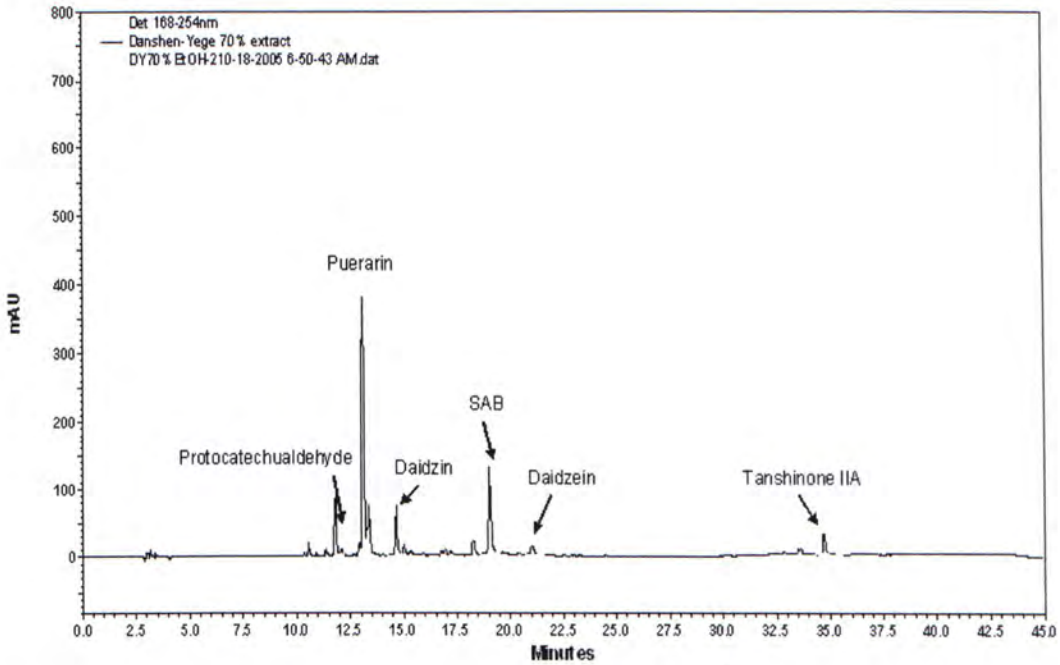


Fig. 3.4 HPLC Chromatogram of DY 70 Extract. *The peaks and retention times of the six markers were shown. The detection was monitored at 254nm. One out of three triplicates was shown here.*

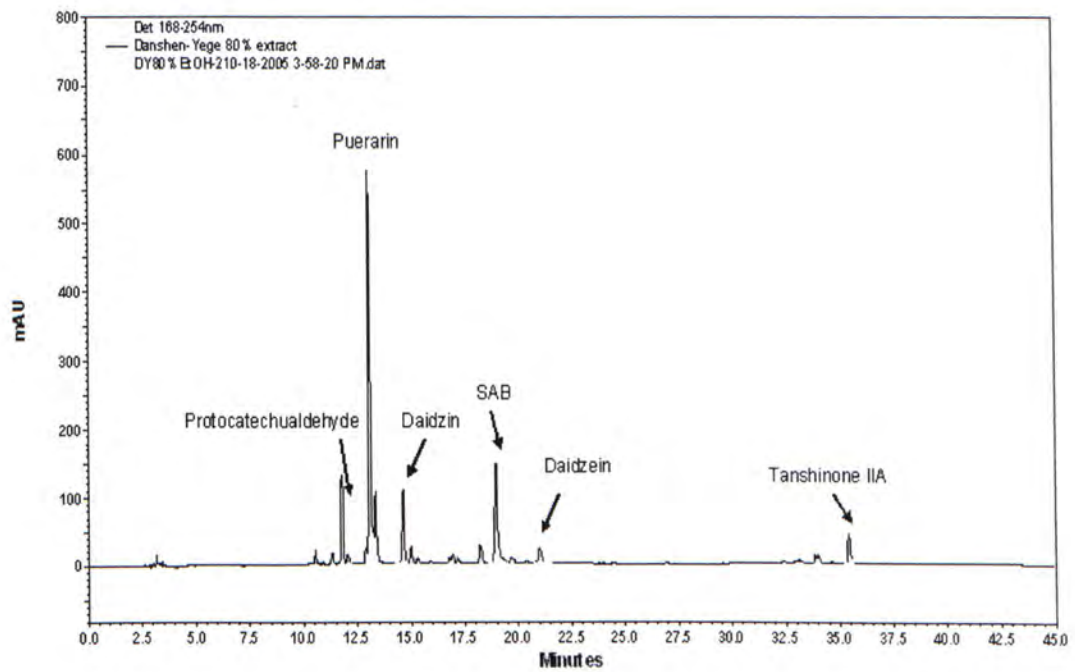


Fig. 3.5 HPLC Chromatogram of DY 80 Extract. *The peaks and retention times of the six markers were shown. The detection was monitored at 254nm. One out of three triplicates was shown here.*

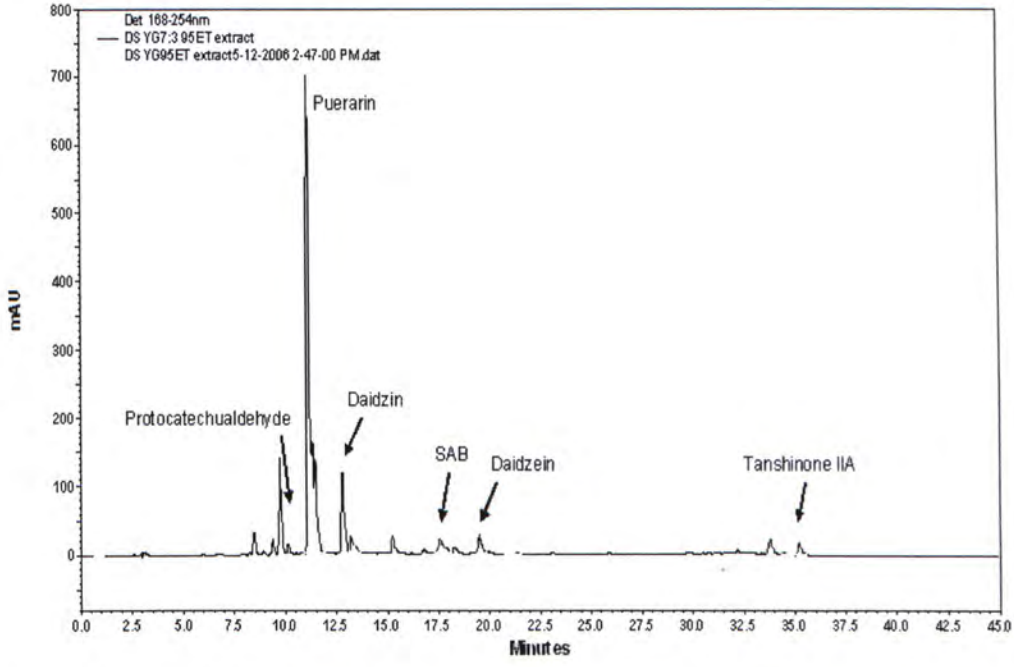


Fig. 3.6 HPLC Chromatogram of DY 90 Extract. *The peaks and retention times of the six markers were shown. The detection was monitored at 254nm. One out of three triplicates was shown here.*

3.1.2.2 Content Percentage of Marker Compounds

The six marker compounds were quantified in various herbal extracts. Contents of the markers in the extracts were estimated by substituting into standard calibration curves. In Table 3.1, puerarin, daidzin, daidzein and tanshinone IIA were increased in extracts obtained from increasing concentrations of ethanol. However, protocatechualdehyde and salvianolic acid B showed a drastic drop in DY 90 extract, in spite of the ethanol concentration(50-80%)-dependent increase.

Markers	Extracts				
	DY 50	DY 60	DY 70	DY 80	DY 90
Protocatechualdehyde	0.047%	0.22%	0.29%	0.44%	0.089%
Puerarin	2.19%	2.4%	3.02%	4.7%	13.67%
Daidzin	0.55%	0.55%	0.7%	1.08%	1.94%
Salvianolic acid B	2.61%	4.62%	5.28%	6.11%	1.33%
Daidzein	0.027%	0.042%	0.081%	0.14%	0.4%
Tanshinone IIA	0.094%	0.14%	0.22%	0.4%	1.2%

Table 3.1 Contents of marker compounds in the different herbal extracts.

The contents were expressed in percentage (w/w). Each data was obtained by taking the average from triplicate trials.

3.1.3 Discussion

The six markers (protocatechualdehyde, puerarin, daidzin, salvianolic acid B, daidzein and tanshinone IIA) used in this HPLC study are the most commonly investigated marker compounds in Danshen and Gegen. From the HPLC analyses of various herbal extracts, the contents of all marker compounds were increased by a higher concentration of ethanol used in the extraction. Yet with the exception in DY 90, as the content of salvianolic acid B and protocatechualdehyde have dropped suddenly. This may be due to the fact that the highly non-polar 90% ethanol solvent was failed to extract these two water-soluble substances. The findings in this part will be further elucidated, after identifying the active components for the vasodilation and antioxidation properties of the Danshen-Gegen formula.

3.2 Studies on Marker Compounds

3.2.1 Introduction

As mentioned earlier, one crucial element in TCM modernization is the understanding of the mechanism of how these herbal medicines work. Chinese medicine has been in use for thousands of years, but the scientific study on its biochemical actions just started in recent decades. Herbal extracts differ from western pharmaceutical drugs in ways that they contain numerous compounds in one plant, whereas the latter is usually composed of one single active chemical. In order to study how an herbal extract works, it is essential to study its pure active compounds. However, a single herb may contain hundreds or thousands of different compounds and they may work together to exert the maximum therapeutic effect. Some compounds were attracting more interest than the others because of their specific chemical structures. For example, phenolic compounds were found to be putative antioxidants due to its capacity to delocalize the electron of free radicals.

Danshen was found to be rich in polyphenolic compounds (Zhang et al., 2005). The most abundant group being the caffeic acid and its derivatives, with biological activities including antioxidant, anti-ischemia reperfusion,

anti-thrombosis, anti-hypertension, anti-fibrosis, antivirus and antitumor properties (Jiang et al., 2005b). On the other hand, previous studies on Yege have reported a number of bioactive isoflavones (Fang et al, 1974). Flavonoids comprise a large group of polyphenolic compounds (Kuehnau, 1976). Most flavonoids have been reported to modulate vascular tone, exert antioxidation effect and reduce LDL-cholesterol (Chan. et al, 2000; Ajay et al, 2003). Other biological activities of flavonoids include anti-inflammatory (Rahman et al., 2003), anti-angiogenic, anti-tumor(Jung et al., 2003) and vessels relaxation actions (Ko et al., 2003) were shown.

In Chapter 2, the optimized 80% ethanol extract of Danshen-Yege has been determined. In order to elucidate the mechanism underlying its vasodilation and antioxidation effects, six pure compounds including the phenolic and isoflavonoid compounds from Danshen and Yege were investigated in the present study. The six pure compounds, including three from Danshen and three from Yege, namely salvianolic acid B, protocatechualdehyde, tanshinone IIA, and puerarin, daidzein and daidzin, were utilized to identify the active components for the Danshen-Gegen formula. Their concentrations in DY 80 extract has been identified in the previous section and were shown in table 3.2.

Drugs	Abbreviation	Molecular Size	Content % in DY 80
Salvianolic acid B	SAB	718	6.11%
Protocatechualdehyde	PC	138	0.44%
Tanshinone IIA	TIIA	294	0.4%
Puerarin	PUE	416	4.7%
Daidzein	DE	254	0.14%
Daidzin	DZ	416	1.08%

Table 3.2 Content Percentages of Marker Compounds in DY 80. Abbreviations and molecular sizes were shown.

3.2.2 Methods and Materials

3.2.2.1 Source of Pure Compounds

Protocatechualdehyde (3,4-dihydroxybenzaldehyde, 25g), puerarin (100mg), daidzein (25mg) were supplied by Sigma. Daidzin (5mg) was supplied by Fluka and tanshinone IIA was supplied by National Institute for the Control of Pharmaceutical and Biological Products (中國藥品與生物制品檢定所).

3.2.2.2 Purification and Identification of SAB

Isolation of SAB

Dried root of Danshen (1 Kilogram) was sliced and boiled with distilled water under reflux at 100°C for 3 times, each for 2 hours and 2 liters of solvent. The decoction was cooled and filtered. The filtrate was then concentrated by rotor evaporator into 500ml solution. Absolute alcohol was added into the concentrated extract to make a final concentration of 70% ethanol mixture. Undissolved residue was precipitated and the mixture was filtered. The clear filtrate was then evaporated again to remove the ethanol and the residue was suspended by distilled water. The extract was then lyophilized into powder.

A column (3.5cm x 45cm) with Macro-reticular resin D101 was washed with distilled water. The extract powder was dissolved by water and chromatographed on the column. Water was applied to elute the inorganic salts and polysaccharides. Then, 50% ethanol was eluted to obtain the yellowish fraction which contains SAB. The fraction was further applied to liquid chromatography of a Sephadex LH-20 column. Successive increase of methanol percentage (20%, 40%, 60% and 70%) in water eluted four fractions DS-1(1.1g), DS-2 (200mg), DS-3 (260mg) and DS-4 (240mg).

Identification

The collected fractions were subjected to Thin Layer Chromatography (TLC) and 1H Nuclear Magnetic Resonance (NMR) for identification. For TLC, the silica gel TLC plate was used, with the mobile phase of Toluene: Ethyl acetate: Formic acid (4:4:1). Fraction DS-1 was identified as Salvianolic acid B, with a molecular size of 718.

3.2.2.3 Vasodilation model

In purpose of identifying the active compound(s) in DY 80, for its vasodilative action, the same *ex vivo* assay, as described in Section 2.3.4, was used. The concentration of pure compounds administered into the organ bath was based on their content in DY 80 (table 3.3).

Drugs	Content % in DY 80	Concentration in Organ Bath (μg/ml)	Equivalence in mM	Solvent
DY80	-	4000	-	Krebs
SAB	6.11%	245	0.34	Krebs
PC	0.44%	18	0.13	Krebs
TIIA	0.4%	16	0.054	0.3% DMSO
Puerarin	4.7%	188	0.45	0.3% DMSO
Daidzein	0.14%	5.6	0.023	0.3% DMSO
Daidzin	1.08%	43	0.104	0.3% DMSO

Table 3.3 Concentrations of pure compounds used in the vasodilation study of DY 80.

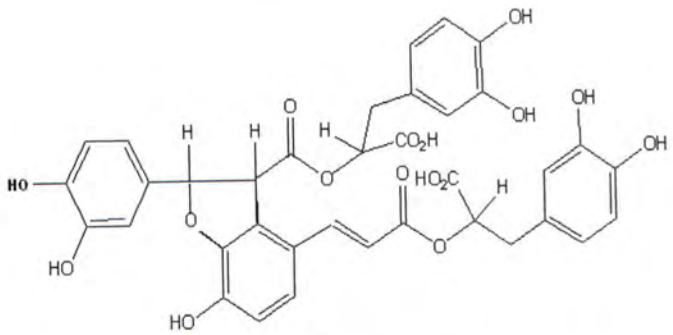
3.2.2.4 Antioxidation Model

Similarly, in order to investigate the active component(s) for the antioxidation action of DY 80, the same *in vitro* antioxidation assay, as mentioned in Section 2.3.3, was used in this study. Concentration of the pure compounds used was estimated according to their content in DY 80 (table 3.4). Successive two fold dilutions were performed to obtain the dose response.

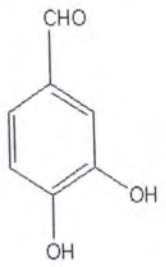
Drugs	Content % in DY 80	Concentration of the highest dosage (μ g/ml)	Equivalence in mM	Solvent
DY80	-	1000	-	PBS
SAB	6.11%	61	0.085	PBS
PC	0.44%	4.4	0.032	PBS
TIIA	0.4%	4	0.014	2.5% DMSO
Puerarin	4.7%	47	0.113	2.5% DMSO
Daidzein	0.14%	1.44	0.006	2.5% DMSO
Daidzin	1.08%	10.8	0.026	2.5% DMSO

Table 3.4 Concentrations of pure compounds in the AAPH-induced RBC Hemolysis Study of DY 80.

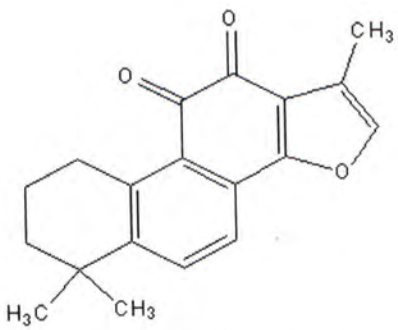
3.2.2.5 Structures of Pure Compounds



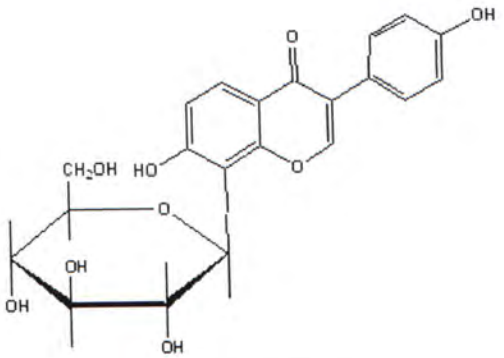
Salvianolic acid B



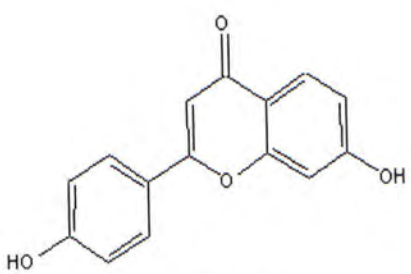
Protocatechualdehyde



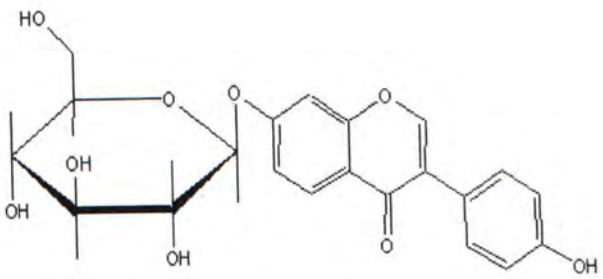
Tanshinone IIA



Puerarin



Daidzein



Daidzin

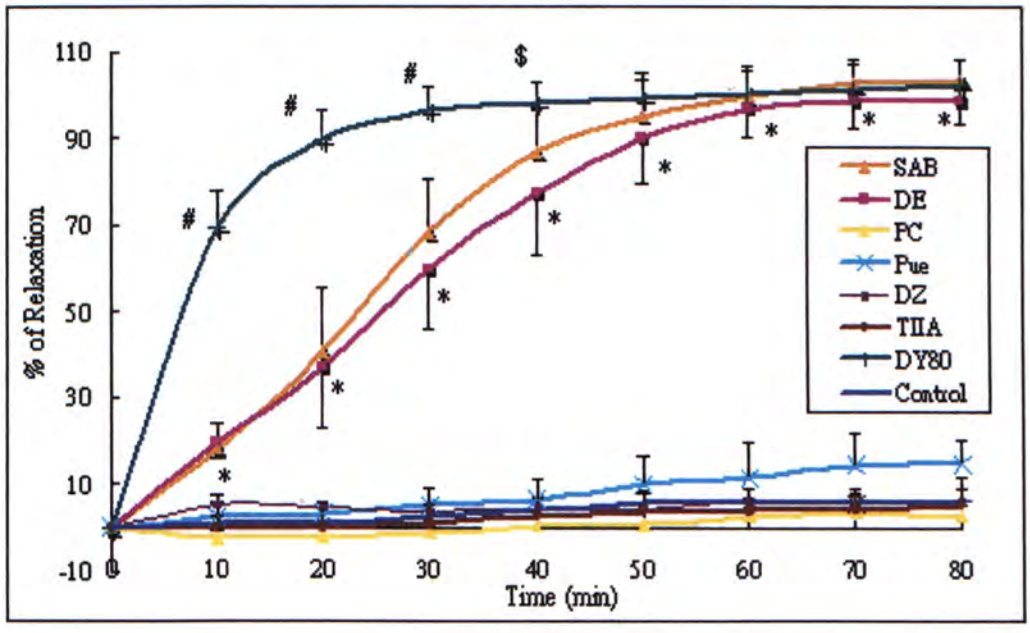
3.2.3 Results

3.2.3.1 Vasodilation Results

Vasodilation responses of pure compounds, which were administered at concentration comparable to their contents in DY 80, were shown in figure 3.7. Among the pure compounds tested, only salvianolic acid B (SAB) and daidzein exerted very potent vasodilative effect. Vasodilating effect of daidzein was especially significant because of its very low concentration administered (5.6ug/ml, equivalent to 23uM) compared with that of SAB (245ug/ml, equivalent to 340uM). Both SAB and daidzein contributed to more than half of the vasodilatory effect of the crude extract DY 80. Puerarin, although showed a relaxation response of the vascular tone for 10%, this was not statistically significant compared to the control, i.e. without drug.

Since the highest physiological active concentration of drugs used in western medicine for this aorta relaxation model is 0.1mM, pure compounds showing no effect were further subjected to the test again at 0.1mM. Among the pure compounds tested, only daidzein and Tanshinone IIA were at concentration lower than 0.1mM. As daidzein was shown to have potent effect, only Tanshinone IIA was further tested at 0.1mM (figure 3.8). However, no

vasodilation effect was observed even at this dosage, therefore it was regarded as a non-active compound. In conclusion, only SAB and daidzein were identified as active compounds for the vasodilative effect of the optimized Danshen-Gegen formula.



n=6

Fig. 3.7 Vasodilatory Effect of Pure Compounds on U46619-precontracted aorta rings. *Results were expressed in percentages of relaxation of the precontracted tone in time-course experiment. The six pure compounds, salvianolic acid B (SAB), daidzein (DE), protocatechualdehyde (PC), puerarin (PUE), daidzin (DZ) and tanshinone IIA (TIIA), were tested with concentrations calculated in table 3.3. Data were shown as mean ± SD (n=6).* $^{*}P<0.001$*, SAB, DE & DY80 versus control.* $^{\#}P<0.001$*, DY80 versus SAB & DE.* $^{\$}P<0.01$ *DY80 versus DE. Only SAB and DE showed significant vasodilatory effect.*

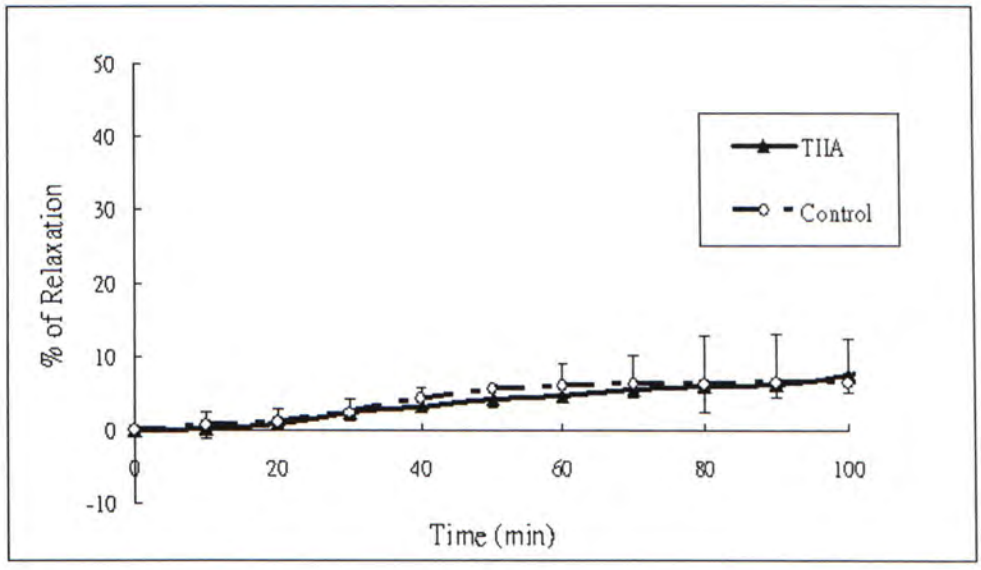


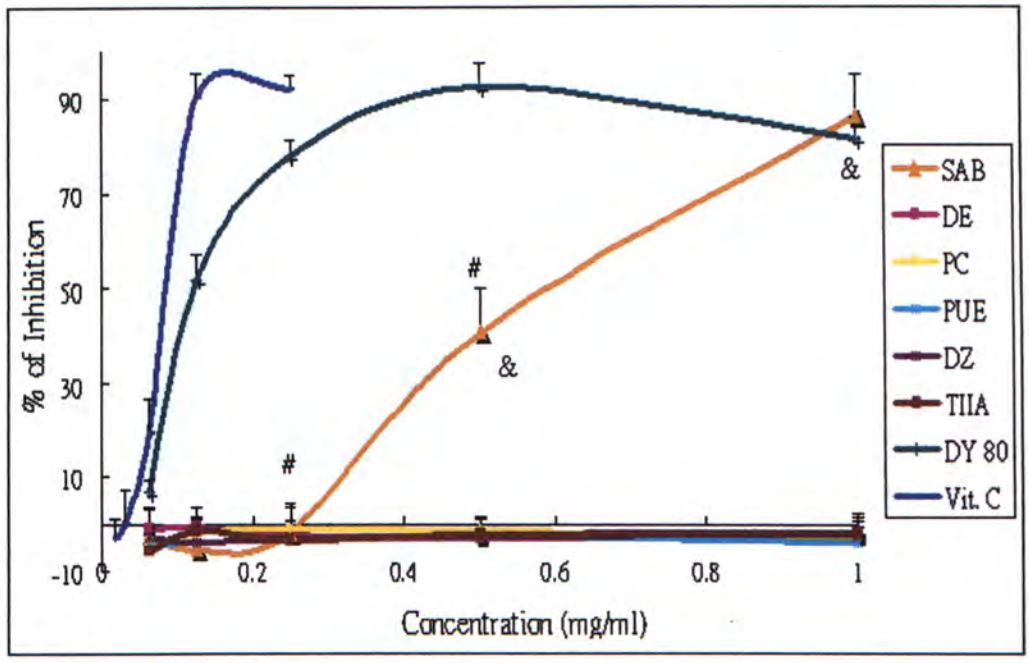
Fig. 3.8 Vasodilation Study of Tanshinone IIA at 0.1mM concentration.

Results were expressed in percentage of relaxation of the precontracted tone in time-course experiment. Tanshinone IIA was applied to organ bath at maximum physiological active concentration of 0.1mM. Data were shown as mean ± SD (n=4). No vasodilatory response was shown compared to control.

3.2.3.2 Antioxidation Results

In order to identify the active compound(s) for the antioxidant effect of DY 80, the six pure compounds were subjected to the antioxidation test, with concentrations comparable to their contents in DY 80 (table 3.4), and their effects were compared with that of DY 80 (figure 3.9). Among the six pure compounds tested, only Salvianolic acid B showed a potent effect on inhibition of hemolysis, with an IC50 of 40.2μg/ml (equivalent to 56μM). Other five compounds did not show any effect at the tested concentrations.

However, the antioxidant effect of SAB was found contributing to less than half of that produced by the crude extract DY 80 (figure 3.9), implying the possibility that other compounds present in DY 80 may account for the rest of the effect. Since the added concentrations of the pure compounds might be underestimated, compounds showing no effect were further studied at the highest concentration of 1mM for the antioxidant effect. The results indicated that three other compounds exhibited antioxidant activities (figure 3.10). Among them, Protocatechualdehyde (90% Inhibition) showed the strongest antioxidant effect, followed by Puerarin (30% Inhibition) and Tanshinone IIA (20% Inhibition).



n=10

Fig. 3.9 Inhibitory Effect of Pure Compounds on AAPH-induced Red Blood Cell Hemolysis. *Results were expressed in the percentage of inhibitory effect of the pure compounds on AAPH-induced red blood cells hemolysis. Pure compounds were tested according to their concentrations in DY 80 calculated in table 3.4 and their inhibitory effects were shown under the corresponding concentrations of DY 80. Concentrations showing in the graph represent that of DY80 and vitamin C only. Vitamin C was used as a positive control. Data were expressed as mean ± SD (n=10).* $^{\&}P<0.001$*, SAB versus other pure compounds.* $^{\#}P<0.001$*, DY 80 versus SAB.*

IC50 values of DY80, Salvianolic acid B and Vitamin C were 112, 40.2 and 89.5μg/ml respectively.

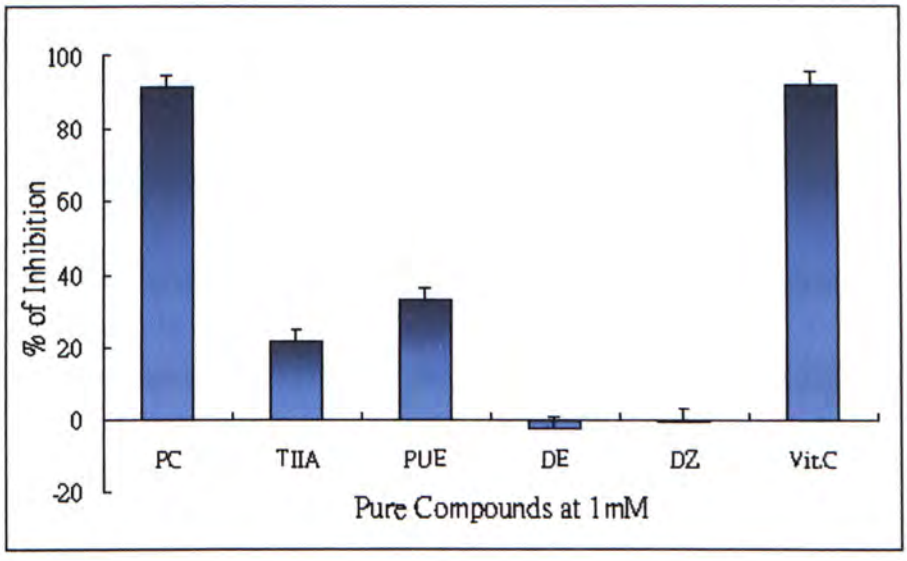


Fig. 3.10 Inhibitory Effect of Pure Compounds at 1mM on AAPH-induced Red Blood Cell Hemolysis. *Results of pure compounds at the highest concentration of 1mM were shown. Data were expressed as mean ± SD (n=5). Protocatechualdehyde, tanshinone IIA and puerarin showed inhibitory effect on hemolysis at this concentration. IC 50 values of protocatechualdehyde and vitamin C were 96uM and 528uM respectively.*

3.3 Discussion

From the present study, salvianolic acid B (SAB) and daidzein were identified as the two major active compounds present in Danshen-Gegen formula. SAB is present in Danshen. It has long been identified to exhibit cardioprotective functions. Antioxidant or radical-scavenging effects have been extensively studied (Fung et al., 1993; Huang & Zhang, 1992; Wu et al., 1998). SAB possesses unsaturated rings with multiple phenolic hydroxyl groups in its structure, which is a common feature in antioxidants and may account for its antioxidant activity. On the other hand, the vasodilatory function of SAB was recently reported (Kamata et al., 1993). Our findings confirmed the same results, and identified this pure compound to be the active component of our formula. Besides inducing vasodilation, SAB is also beneficial to the prevention of other atherogenic events. These include its protective action against TNF-alpha injury in human aortic vascular endothelium cells by reducing cell adhesion molecules VCAM-1 and ICAM-1, which recruits leukocytes (Chen et al., 2001; Chen et al., 2001), improving blood circulations and renal functions (Yokozawa et al., 1991), antifibrotic activity (Shigematsu et al., 1994), reducing myocardial damage in an ischemia-reperfusion model (Fung et al., 1993), inducing apoptosis in neointima, which help prevent the

neointimal thickening (Hung et al., 2001), inhibiting VEGF-induced hyperpermeability (Qui et al., 2001) and antihypertensive effect which may be related to ACE inhibitory property (Kang et al., 2003).

Regarding daidzein, previous studies have been focused on its beneficial effects on postmenopausal women as an estrogen-analog supplement. Yet, only a few studies have reported its vessel relaxant effects (Mishra et al., 2000; Karamsetty et al., 2004). Daidzein belongs to a group of compounds called isoflavones; the latter form a part of the group phytoestrogens. Phytoestrogens are polyphenolic non-steroidal plant compounds which have biological activities similar to estrogen 17beta-estradiol (Murkies et al., 1998) and may exhibit selective estrogen receptor modulating activities due to their similar structures. Studies showed that high consumption of phytoestrogen has protective effects on cardiovascular diseases (Bakhit et al., 1994; Teede et al., 2001) and lowers the total and LDL-cholesterol level (Crouse et al., 1999; Gardner et al., 2001), also exhibits beneficial effects in osteoporosis (Potter et al., 1998) and cancers (Lee et al., 1991). Among the groups of phytoestrogens, isoflavones is the most studied one. Daidzein and puerarin from Gegen belong to this group. Isoflavones has been reported to reduce plasma cholesterol, probably by up-regulating LDL-receptors, and hence reducing atherosclerosis

(Kirk et al., 1998). Antioxidant activity of isoflavones has also been reported (Rufer & Kulling, 2006). However, in this study, no antioxidant activity of daidzein has been shown.

Daidzein may act like estrogen, and thereby prevent atherosclerosis. Previous study has demonstrated that Yege has estrogenic activities (Zhang et al., 2005). The action of estrogen has been extensively studied. It was known to decrease the risk of coronary disease in postmenopausal women (Nabulsi et al., 1993), increase HDL cholesterol and decrease LDL cholesterol *in vivo* (Walsh et al., 1991), inhibit SMC proliferation and migration (Suzuki et al., 1996), increase NO production and eNOS protein expression (Hayashi et al., 1995). Direct action of estrogen on vessel walls has been reported, and estrogen receptors were found in the vascular endothelium and smooth muscle cells. It is possible that daidzein may act on these estrogen receptors to exert its function. Besides, it has been reported that the majority of daidzein present in plant is in the form of inactive glycoside Daidzin and derivative biochanin A. It was reported that these precursors can be metabolized in the digestive tract by enzymes into the active form daidzein (Day et al., 1998). If this is the case, the beneficial effect of this formula might be enhanced by a higher content of Daidzin detected in the extract, which may be converted into the active form

daidzein *in vivo*. Other cardioprotective functions of daidzein reported include the reduction in monocyte chemoattractant protein-1 (MCP-1) secretion induced by TNF-α in human umbilical vein endothelial cells (Gottstein et al., 2003) and anti-thrombotic effects (Choo et al., 2002).

Besides SAB and daidzein, previous research reported that the activity of other pure compounds present either in Danshen or Yege may be beneficial in inhibiting atherogenesis. For pure compounds of Danshen, rosmaric acid were found to lower the production of superoxide anion radical ($O_2^{\cdot-}$) in the xanthine oxidase system, showing anti-thrombotic and antiplatelet effects (Zou et al. , 1993). Tanshinones were found to inhibit platelet aggregation, protect myocardium against ischemia-induced derangements (Lin et al., 2001; Yagi et al., 1989) and inhibit inflammatory responses (Kim et al., 2002). Protocatechualdehyde was found to selectively inhibit cytokine-induced VCAM-1 and ICAM-1 expression and reduce monocyte adhesion to endothelium cells through actions involving NF-κ B and AP-1 (Zhou et al., 2005). Other polyphenolic compounds were reported to exert antioxidant, anti-ischemic reperfusion, anti-thrombosis, anti-hypertension and anti-fibrosis actions(Jiang et al., 2005a). In contrast, less research has been done on active compounds in Yege compared with those in Danshen. Yet, some major

compounds have been identified. Genistein was found to reduce plasma homocysteine level, prevent oxidation (Chen et al., 2005) and inhibit the occlusion of thrombotic vessel (Kondo et al., 2002). Puerarin decreases serum total cholesterol (Yan et al., 2006b), inhibits arrhythmias (Chai et al., 1985), suppresses proliferation of vascular smooth muscle cells (Xu et al., 2006) and induces endothelium-independent relaxation (Dong et al., 2004). However, our study did not show any vasodilating property of puerarin, although it is the most abundant isoflavones in Gegen.

After identifying SAB as a potent vasodilator and antioxidant, and daidzein as a potent vasodilator, the results of previous studies could be further elucidated here. As elaborated in the previous chapter, increasing the ethanol percentage in the extraction process up to 80% enhanced the vasodilative and antioxidative activities, but antioxidant effect of the extract markedly reduced when 90% ethanol was used. HPLC analysis indicated that highly non-polar solvent (90% ethanol) failed to dissolve and extract polar compounds, protocatechualdehyde and SAB, and hence resulted in a drop in the contents of these water-soluble substances in the extract. Since SAB and protocatechualdehyde were identified as the two major pure compounds account for the antioxidant activity of DY extract, the drop in their content may

explain the reduction in antioxidant effect of DY 90. Although SAB content in DY 90 was reduced, the increase in daidzein content, another more potent vasodilator, may help compensate for the loss and resulted in an overall greater vasodilation effect of DY 90 than other extracts. However, among the extracts, DY 80 had the most holistic and balanced amount of active compounds in proportion and in optimal contents, thus possessing potent effects in both antioxidant and vasodilatory activities.

3.4 Synergistic Effect Study

3.4.1 Introduction

As mentioned previously, different compounds in an herbal extract may work together to enhance activities. Since two pure compounds have been identified, one (SAB) present in Danshen and one (daidzein) present in Gegen, both of which contribute to much of the vasodilating effect demonstrable in the crude extract (DY 80), a question arises if these two compounds could produce synergistic effect on antioxidation and vasodilation.

3.4.2 Methods

The same vasodilation assay model, as described in Chapter 2, was used. The concentrations of SAB and daidzein used were the same as in the previous section, i.e. 245 and 5.6 μ/ml, respectively. The two pure compounds were combined before administering to the precontracted aorta rings suspended in organ baths.

3.4.3 Results

SAB and daidzein were examined for synergistic effect on the vasodilation action. The combination of SAB and daidzein resulted in a significantly higher vasodilative effect as compared with that of the individual active compound (figure 3.11). However, the effect of this combination did not outperform the effect of the summation of SAB and daidzein. And the effect of this combination of SAB and daidzein could not completely account for the effect of DY 80, as a significant difference in response was shown at 10 minutes after the administration of drugs.

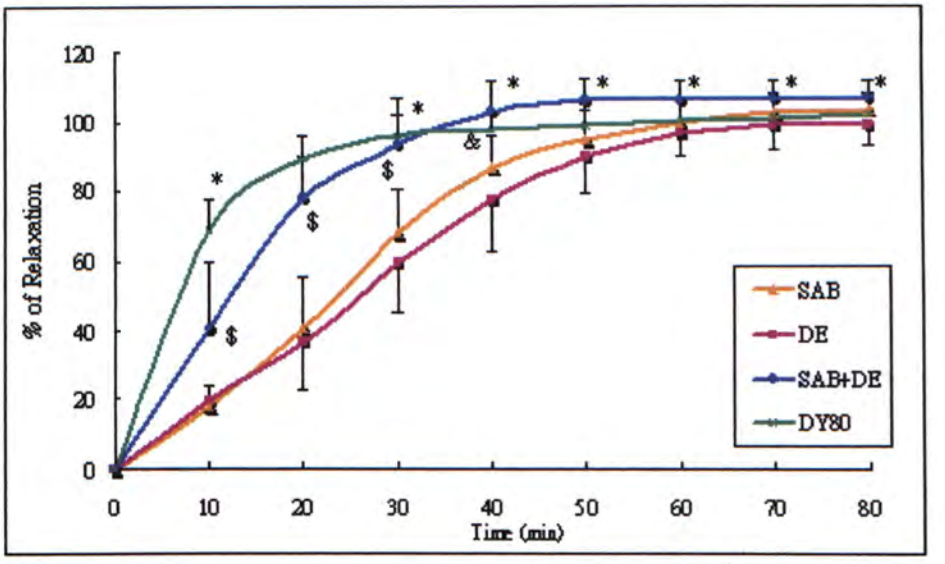


Fig. 3.11 Synergistic Study of Salvianolic acid B and Daidzein on Vasodilation Effect. *Results were expressed in percentages of relaxation of the precontracted tone in a time course experiment. SAB and daidzein were combined just before adding into the precontracted aortic rings. Data was shown in mean ± SD (n=7).* $^{\$}P<0.01$*, SAB+DE versus SAB & DE.* $^{\&}P<0.05$*, SAB+DE versus DE.* $^{*}P<0.05$*, DY 80 versus SAB+DE. The maximum difference in the relaxation response between the combination of SAB and daidzein with SAB and daidzein alone were 37.13 ± 5.7% and 40.9 ± 3.27 respectively.*

3.4.4 Discussion

Combination of SAB and daidzein was shown to exert an additive effect, which means the outcoming response was approximately equal to that of the summation of the individuals. The additive action was shown to be close to that of the crude extract, and hence SAB and daidzein were likely to contribute mainly to the vasodilatory activity of DY 80. The possible presence of other active compounds in DY 80 may explain the rest of the vasodilative effect detectable in DY 80.

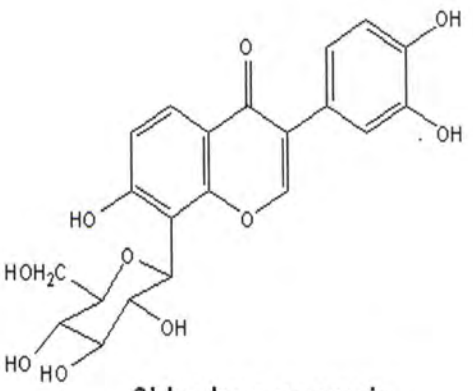
The present study did not show synergistic effect between SAB and daidzein. This may be due to the fact that the two active compounds were combined shortly just before adminstered to the aortic rings, the time might be insufficient for the compounds to interact and enhance each other's activity. Longer time and heating may be needed to micmic the boiling effect during extraction process and let the compounds to interact to generate possible synergistic effect. Synergistic interactions of pure compounds are of vital importance in herbal medicines, which may also provide an explanation for the efficacy of apparently low doses of active constituents in an herbal extract. Synergistic interactions among different constituents within a single herb, as

well as between different herbs in a formulation were documented (Williamson, 2001). It is possible, but not shown in our results that the presence of one compound may somehow enhance the drug bioavaliability and biological action of the other, so as to exaggerate the vasodilation effect. The findings in the present study demonstrated an additive but not synergistic effect between the compounds from two different herbs, which provides evidence that the compound formula of Danshen and Gegen can outperform the single herb. And this result implied that the pure compounds, SAB and daidzein, may act through different mechanisms to induce vasodilation. The mechanisms underlying the pharmaceutical actions of these two compounds were studied, as described in later chapters.

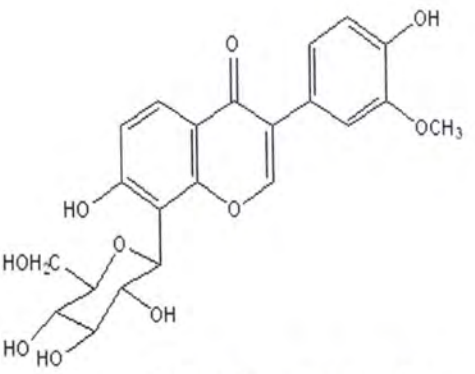
3.5 Study on 3'-hydroxypuerarin and 3'-methoxypuerarin purified from Yege

3.5.1 3'-hydroxypuerarin and 3'-methoxypuerarin

Apart from the more commonly studied pure compounds investigated in previous section, 3'-hydroxypuerarin and 3'-methoxypuerarin are two other pure compounds which have been identified in Yege, but absent in Fenge (Jiang et al., 2005b). These two pure compounds may contribute to the difference in the bioactivity reported between these two types of Gegen. But so far no previous studies have reported the cardiotonic effect of these two pure compounds. Therefore, we propose to purify and study these two compounds in the herbal formula extract.



3'-hydroxypuerarin



3'-methoxypuerarin

Fig. 3.12 Structures of 3'-hydroxypuerarin and 3'-methoxypuerarin. *The two compounds are derivatives of puerarin. They are similar in structures, with a difference at C3, as 3'-hydroxypuerarin possess a hydroxyl group while 3'-methoxypuerarin possess a methoxyl group at this position.*

3.5.2 Methods and Materials

3.5.2.1 Purification by HPLC semi-preparation

Isolation

3'-hydroxypuerarin and 3'-methoxypuerarin were purified by semi-preparative HPLC. Dried root of Yege (50g) was sliced and extracted with distilled water at room temperature in ultrasonic water bath for one hour, two times. The extracted solutions were pooled together and concentrated by

rotor evaporation under reduced pressure to yield dried powder (5.1g). The extract of Yege was then dissolved in 15% Acetonitrite (ACN) to give a 100mg/ml solution. The solutions were filtered through a 0.45μm filter before transferring to autosampler vials ready for HPLC purification. The semi-preparative HPLC of Yege extracts was performed in the Beckman Ultrasphere ODS, 5u, 10mm x 250mm column, with detection by Beckman System Gold 508/125/168. Using a mobile phase of 85% A: 0.2% acetic acid and 15%B: ACN. The flow rate was maintained as 1ml/min and detection wavelength was set at 254nm. Fractions containing the two pure compounds were collected respectively. The solvent was then evaporated and the pure compound powders were obtained by lyophilization.

Identification

The yield of 3'-hydroxypuerarin and 3'-methoxypuerarin were 5.8mg and 4.9mg respectively. These two pure compounds purified were identified as 3'-hydroxypuerarin and 3'-methoxypuerarin by 1H-NMR and ESI-MS. Molecular size of 3'-hydroxypuerarin and 3'-methoxypuerarin are 432 and 446, respectively.

3.5.2.2 Bioassays

The same protocols for vasodilative and antioxidant properties, as described in Chapter 2, were used in the present study. The concentrations of 3'-hydroxypuerarin and 3'-methoxypuerarin were administered at their maximum physiological active of 0.1mM into organ baths, respectively, for the vasodilative function test. In advance of investigating the antioxidative activities of 3'-hydroxypuerarin and 3'-methoxypuerarin, the antioxidant effect of 80% ethanol extract of Yege was first verified. The 80% ethanol extract of Yege was prepared by the same extraction method, as described in chapter 2, and subjected to antioxidation assay at the highest concentration of 2mg/ml.

3.5.3 Results

3.5.3.1 Vasodilation Study

Purified 3'-hydroxypuerarin and 3'-methoxypuerarin were subjected to the vasodilation assay. However, when compared with the control (0.025% DMSO) without drug, both 3'-hydroxypuerarin and 3'-methoxypuerarin did not show any significant vasodilating activity.

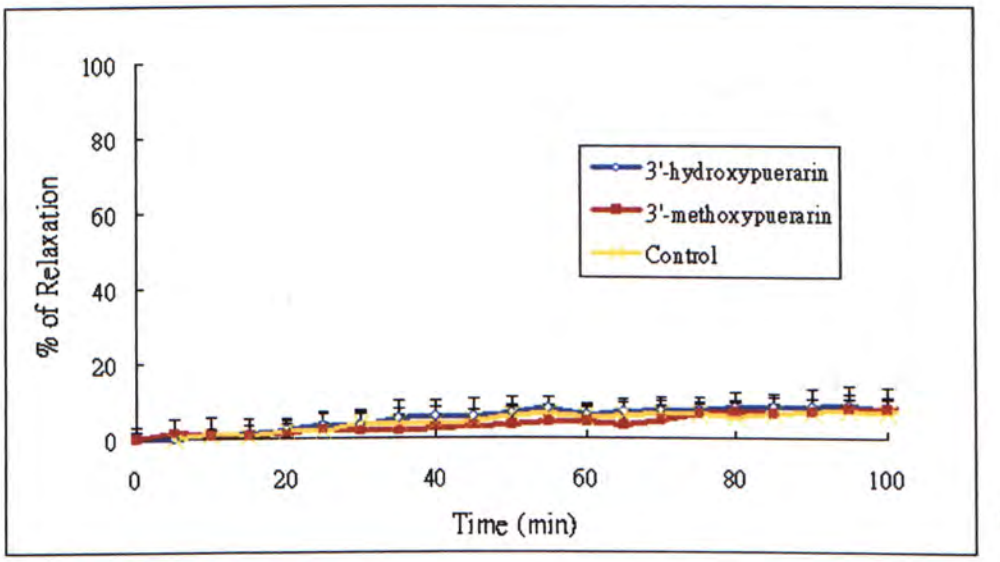


Fig. 3.13 Vasodilative Study of 3'-hydroxypuerarin and 3'-methoxypuerarin on U46619-precontracted rat aorta rings. *Maximum physiological active concentration of 0.1mM of 3'-hydroxypuerarin and 3'-methoxypuerarin were administrated to aortic rings respectively. Data were expressed as ± SD (n=5). No vasodilative effect was shown.*

3.5.3.2 Antioxidative Effect of Yege

80% ethanol extract was prepared and subjected to the antioxidation assay. The extract at different concentrations was studied to obtain the response curve. The results showed that this extract exerted a maximum inhibitory effect of approximately 50% inhibition on cell hemolysis at 1mg/ml concentration.

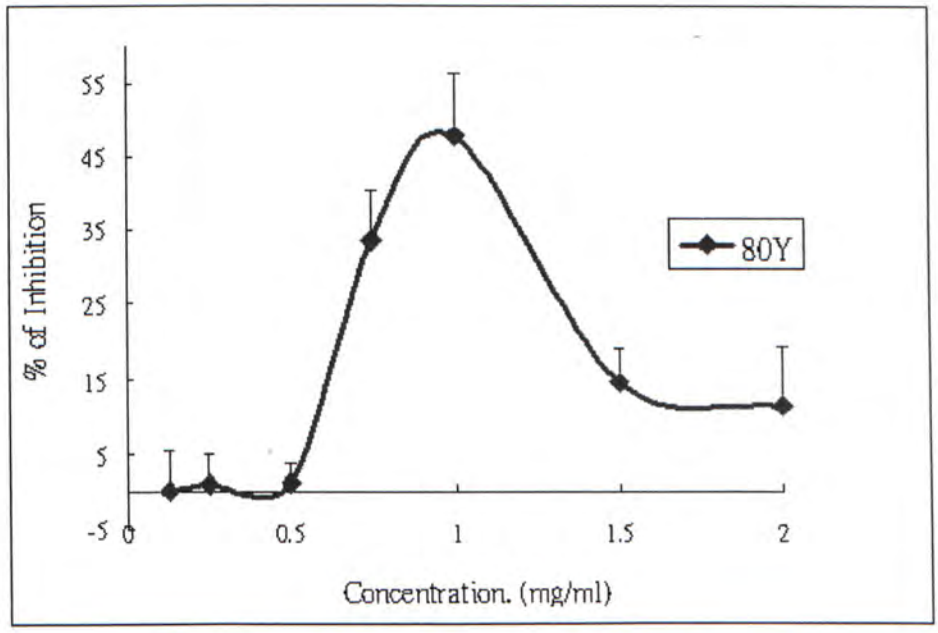


Fig. 3.14 Inhibitory Effect of 80% ethanol extract of Yege on AAPH-induced RBC Hemolysis. *The results were expressed as the percentage of inhibitory effect on AAPH-induced red blood cells hemolysis. Data were expressed as mean ± SD (n=4). Maximum inhibitory effect was shown at 1mg/ml for 47.7 ± 8.5 %.*

3.5.4 Discussion

Since 3'-hydroxypuerarin and 3'-methoxypuerarin are present in Yege, but not in Fenge (Jiang et al., 2005b). These two pure compounds may somehow contribute to the pharmacological difference between the two species of Gegen. The two compounds purified were first subjected to vasodilative functional study. However, no vasodilating effect was shown. Therefore, these two compounds are not likely to account for the more potent vasodilative effect of Yege compared with that of Fenge. The greater vasodilative response induced by Yege may solely be contributed by the higher content of daidzein, the active vasodilator, present in Yege (0.14%) compared with that present in Fenge (0.05%) (Jiang et al., 2005b).

These two pure compounds may, however, contribute to the antioxidation property of Yege. Previous study by my colleague (Yam, 2005) has shown no antioxidant activity of Fenge. However, in the present study, the ethanol extract of Yege has showed an inhibitory effect on free radical-induced hemolysis, inferring that besides puerarin (40% inhibition at 1mM), other compounds may contribute to the overall antioxidant effect of Yege. Therefore, it was proposed that 3'-hydroxypuerarin and 3'-methoxypuerarin may exhibit some extent of

antioxidant effect. However, the putative antioxidative property of 3'-hydroxypuerarin and 3'-methoxypuerarin remains to be determined, owning to the insufficient amount of pure compounds prepared. A further study would be needed after the purification of a larger amount of these two compounds.

Chapter 4

Mechanistic Study

4.1 Introduction

Blood vessels are composed of smooth muscle cells and an inner monolayer of endothelial cells, which are crucial in regulating vascular tone. Upon stimulation, constricting or dilating messagers are released from the endothelial cells, acting on the smooth muscle (SMC) cells through different pathways and eventually causing SMC to contract or relax, changing the diameter and blood flow in vessels. The mechanism of actions of DY 80 and the two active compounds, SAB and daidzein, on inducing vasodilation was studied in the present study.

Vasoconstriction

Endothelial cells are capable to release vasoconstricting substances, including prostanoids such as thromboxane A2 and prostaglandin H2, endothelin, vasopressin and superoxide anions. All these substances cause an increase in intracellular calcium level $[Ca^{2+}]_i$ and cause contraction of the smooth muscle.

Vasodilation

Three endogenous vasodilators released by the endothelium include endothelium derived relaxing factor (EDRF/NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF), all subsequently act on the smooth muscle and result in the relaxation.

4.1.1 Nitric Oxide-mediated Vasodilation

Endothelium derived relaxing factor (EDRF), is commonly known as nitric oxide (NO), includes also its closely related nitroso-containing compounds. Substances which cause vasodilation through this pathway increase the production of NO in endothelial cells. Enhanced NO production is mediated by the activation of nitric oxide synthase (NOS) which convert the substrate L-arginine into NO. Once released from the endothelium, NO diffuses through the vascular wall into the smooth muscle cell and activates the cytosolic enzyme guanylate cyclase (GC) to increase cyclic GMP (cGMP) level. This increase in cGMP activates the protein kinase G (PKG), inhibits calcium entry into the SMC, activates K^+ channels, and decreases IP_3, causing relaxation of the smooth muscle cells. There are three isoforms of the enzyme NOS, namely endothelial constitutive NOS (eNOS), inducible NOS (iNOS) and neuronal

NOS (cNOS). eNOS is the most important form of NOS in the cardiovascular system. It is the main enzyme responsible for the continual production and release of NO by the endothelium cells. Many vasodilators were demonstrated to act through this pathway (Furchgott, 1984; De Mey & Vanhoutte, 1981)

4.1.2 Prostacyclin-mediated Vasodilation

Prostacyclin (PGI_2) is produced in endothelial cells from prostaglandin H2 by the action of enzyme prostacyclin synthase. Its action counteracts that of thromboxane, a vasoconstrictor. After releasing from the endothelium, it promotes vasodilation by increasing the cellular cyclic AMP in smooth muscle cells, which in turn activates protein kinase A (PKA). PKA inhibits Ca^{2+} influx into smooth muscle, lowering $[Ca^{2+}]_i$ and thus cause muscle relaxation. Besides vasodilation, the most important role of PGI_2 is limiting platelet attachment and aggregation.

4.1.3 EDHF-mediated Vasodilation

Endothelium-derived hyperpolarizing factor (EDHF) is defined as the non-nitric oxide (NO) and non-prostacyclin (PGI_2) substance that mediates hyperpolarization of vascular smooth muscle cells and cause relaxation. The chemical nature of EDHF is unknown. However, these factors contribute to vasodilation by opening the K^+ channels on SMC, causing hyperpolarization via regulating the calcium gradient and extracellular calcium, resulting in relaxation of smooth muscle. Several types of K^+ channels are expressed on smooth muscle cells, namely Kir, BKca, Kv, etc.

Inwardly rectifying K^+ channels (Kir)

Inwardly rectifying K^+ channels (Kir) is one of the most important channels for the control of the resting potential of cells. These channels are characterized by its ability to allow large inward currents and smaller outward currents. Kir is a K^+ sensor, even a small increase in the extracellular K^+, would increase the conductance of Kir.

High-conductance Ca^{2+}-activated K^+ channels (BKca)

High-conductance Ca^{2+}-activated K^+ channels (BKca) respond rapidly to both increase in intracellular calcium level and voltage, which enhance its opening. Isoforms of this channel include intermediate-conductance channel (IKca) and small-conductance channel (SKca).

Voltage-dependent K^+ channels (Kv)

Voltage-dependent K^+ channels (Kv) are mainly located on smooth muscle cells. It is also regarded as delayed rectifying K^+ channel and responds to voltage changes.

4.1.4 Endothelium-dependent and -independent Vasodilations

As mentioned previously, endothelium is vital in vascular functions which include controlling coagulation, fibrinolysis, vascular tone and growth. Any substance which may restore or increase bioavailability of endothelial products, such as NO, prostacyclin and EDHF, raises the possibility of modifying arterial function and may reverse endothelial dysfunction conditions. Studies have reported the possibility of certain strategies to reverse endothelial dysfunction (Levine et al., 1996; Clarkson et al., 1996). Actions of these potential substances have to be on the endothelium, inducing the release of powerful vasodilators as a consequence and in turn improve the health of the endothelium.

Therefore, endothelium-dependent or -independent vasodilation was determined in the present study by using specific ligands to block enzymes or mediators located on endothelium or smooth muscle cells of vessels. Changes due to removal of endothelium was also studied, which may cause an attenuation of effect induced by that drugs. Drugs targeting on the endothelium for its actions may be more relevant in the prevention of atherogenesis, for their possibility to improve the endothelium and hence the vascular health.

4.2 Methods and Materials

The same assay for studying the vasodilation function, as mentioned in Chapter 2, was used in the present study, to investigate the change in vascular relaxation tone by different inhibitions. Animals, aorta isolation and preparation procedures were performed as described previously.

Denuded aorta rings preparation

Aorta rings (2mm) were subjected to mechanical removal of endothelium by rubbing the aorta lumen with a steel wire. After equilibration and gradually stretched the rings to 1.5g resting tension, 0.3uM of Phe was used to test the normal functioning of smooth muscle layer, followed by Ach. Complete removal of endothelium gave no relaxation in response to Ach. The aorta rings were then washed with warmed Krebs solution to restore tension back to their resting value. The rings were equilibrated again for an hour before precontraction by U46619 and addition of corresponding drugs.

High K^+ solution treatment

KCl (60mM)-containing solution was prepared by substituting NaCl in Krebs solution by an equal molar of KCl to retain constant ionic strength. After testing endothelium integrity and equilibration, aorta rings were suspended in high K^+ solution by replacing 8ml 60mM KCl Krebs solution into organ baths. Aortic contraction was induced, corresponding drugs were added after the appearance of sustained tone.

Blockers treatment

After testing endothelium integrity and equilibration, specific blockers were added into organ baths, with aorta rings suspended for 30 minutes. Then, either 10mM or 15mM U46619 was added to obtain an initial contracted tone similar to that without blockers. Corresponding drugs were then added after the sustained contracted tension. The following table presented the action, concentration and source of different blockers (table 4.1).

Blockers	Full Name	Action	Concentration	Source	U46619
L-NAME	NG-nitro-L-arginine methyl ester	Non-specific nitric oxide synthase inhibitor	100 μM	Sigma	15mM
ODQ	1H-[1,2,4]oxadiazolo-[4,2-alpha]quinoxalin-1-one	Guanylate cyclase inhibitor	3 μM	Sigma	10mM
Indomethacin	-	Non-selective cycloxgenase	1 μM	Sigma	10mM
$BaCl_2$	Barium chloride	Kir channel blocker	0.3mM	Merck	15mM
TEA	Tetraethylammonium chloride	Non-selective BK_{ca} channel blocker	10mM	Sigma	15mM
4-AP	4-aminopyridine	K_v channel blocker	3mM	Sigma	15mM

Table 4.1 Inhibitors used for mechanism study, showing full names, concentration, source and actions.

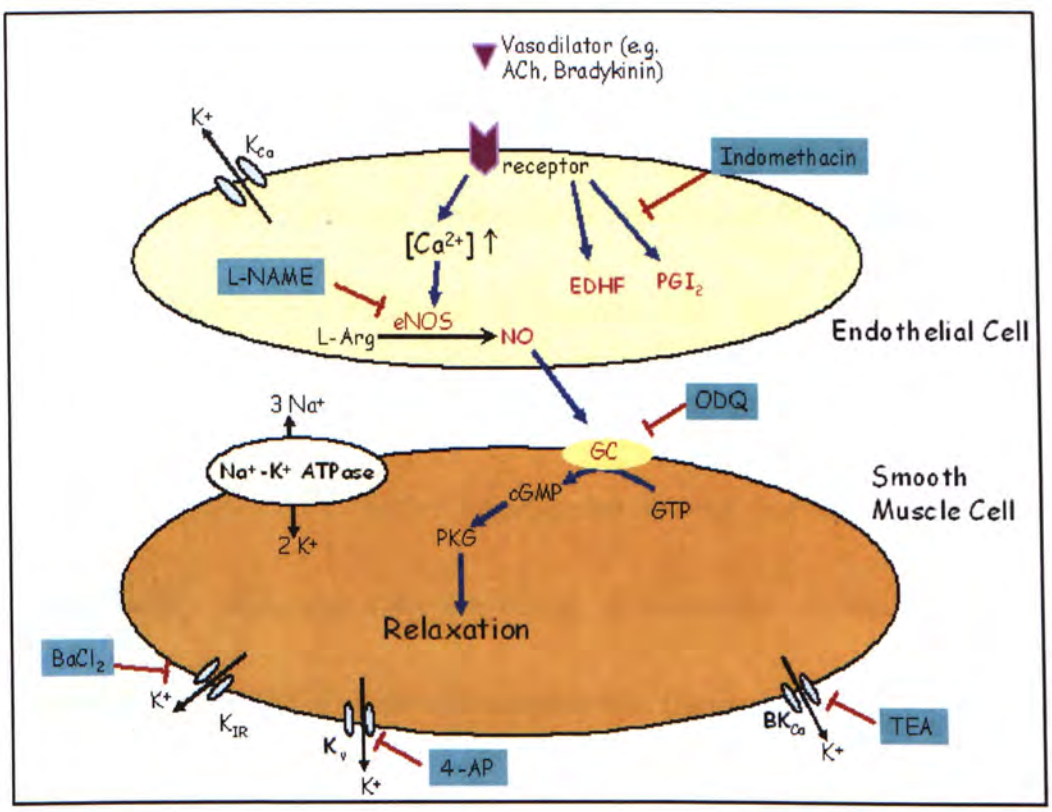


Fig. 4.1 Vasodilation mechanisms and Blockers Effect. Red arrows represent the action of blockers and blue arrows represent activations.

4.3 Results

After the identification of active compounds responsible for DY 80 extract's vasodilating response in the previous section, in the present study, the mechanism underlying the actions of these pure compounds (SAB and daidzein) and the crude extract (DY 80) were investigated.

4.3.1 Danshen-Gegen Formula (DY 80)

According to the results shown in figure 4.2 – 4.5, relaxation tone induced by DY 80 extract remained unchanged in denuded aorta rings and in rings treated with L-NAME, ODQ and indomethacin. However, in the presence of elevated extracellular K^+, the relaxation response of DY 80 was partially abolished (figure 4.6). Further investigation showed that aortic rings treated with $BaCl_2$, TEA and 4-aminopyridine, demonstrated a certain degree of inhibition of the DY 80-induced relaxation tone (figure 4.7 – 4.9).

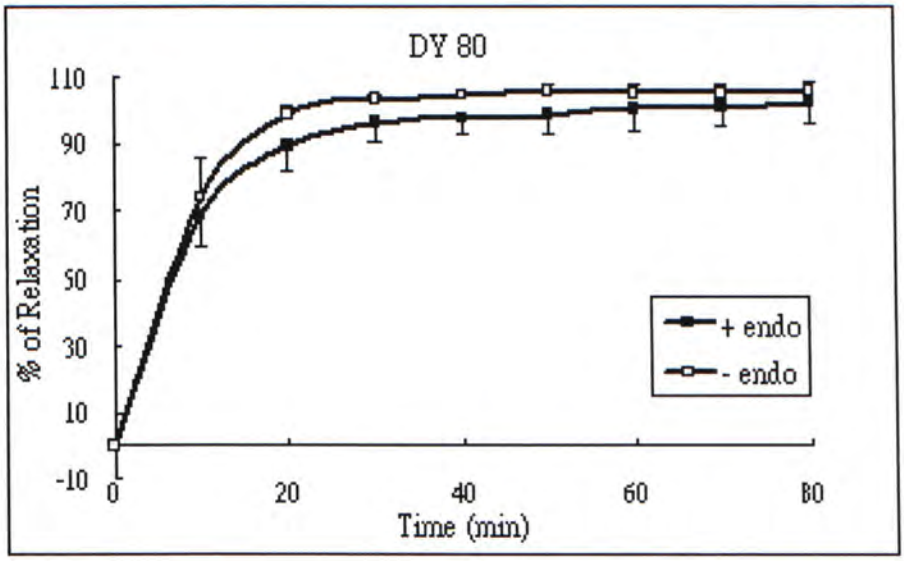


Fig. 4.2 Effect of denuded aorta rings on DY 80-induce relaxation compared with intact rings. *Percentage of relaxation of the pre-contracted tone was compared in the course of time. "+ endo" represents the effect of DY80 on intact aorta rings. "-endo" represents the effect of DY80 on denuded rings. Data were presented as mean ± SD (n= 4). No inhibitory effect was shown.*

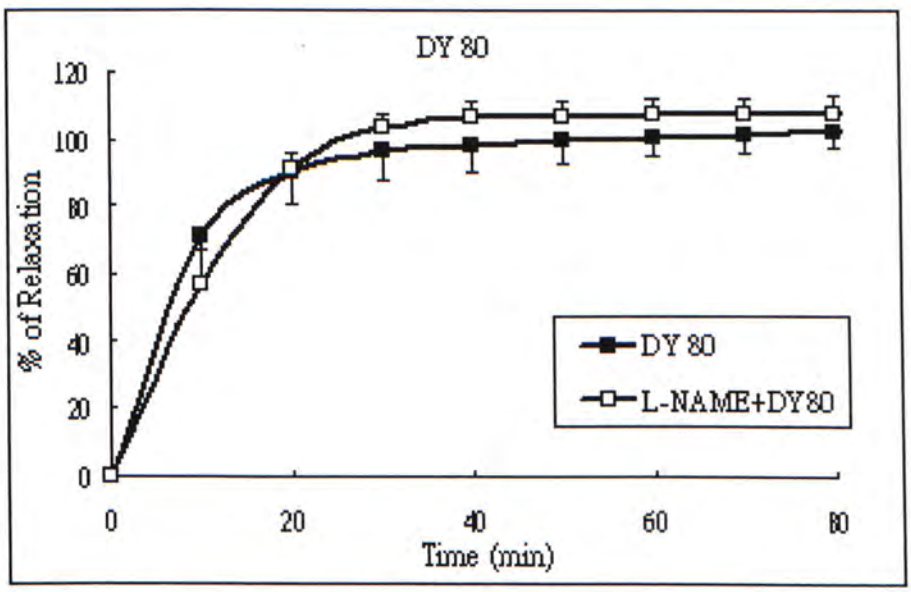


Fig. 4.3 Effect of L-NAME on DY 80-induced relaxation of intact aorta rings. *L-NAME (100 μM) was added 30 minutes prior to the addition of DY 80 (4mg/ml). Data were presented as mean ± SD (n= 5). No inhibitory effect was shown.*

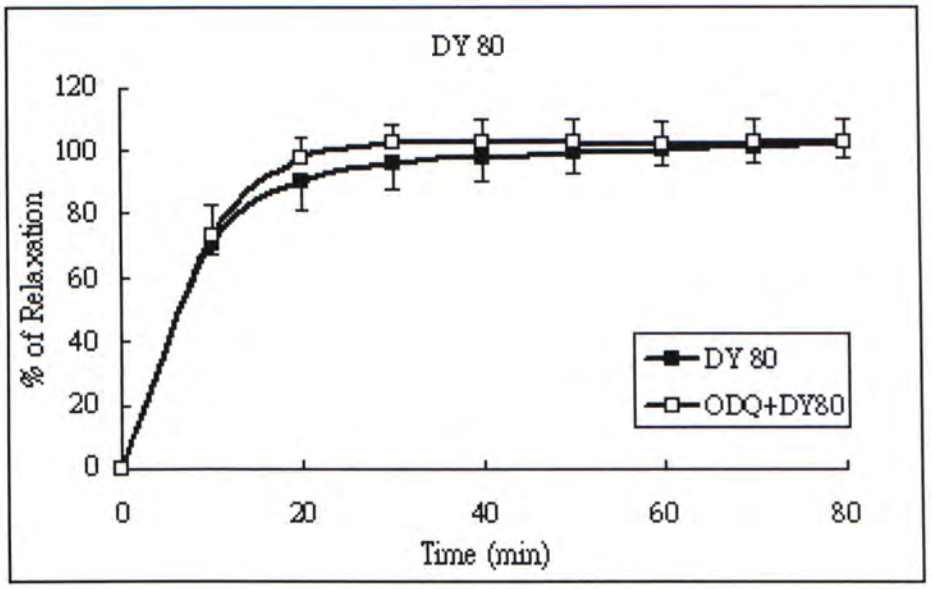


Fig. 4.4 Effect of ODQ on DY 80-induced relaxation of intact aorta rings. *ODQ (3 μM) was added 30 minutes prior to the addition of DY 80 (4mg/ml). Data were presented as mean ± SD (n= 6). No inhibitory effect was shown.*

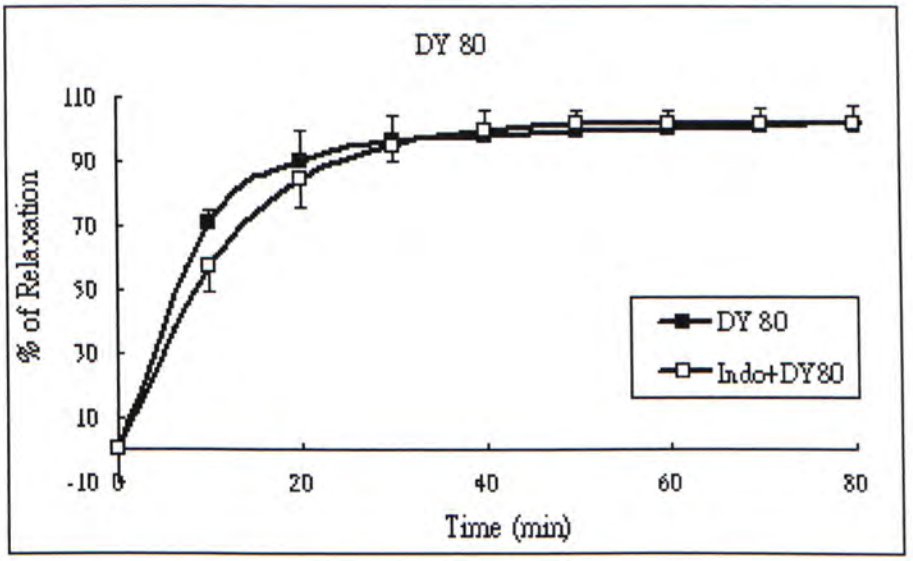


Fig. 4.5 Effect of Indomethacin on DY 80-induced relaxation of intact aorta rings. *Indomethacin (1μM) was added 30 minutes prior to the addition of DY 80 (4mg/ml). Data were presented as mean ± SD (n= 6). No inhibitory effect was shown.*

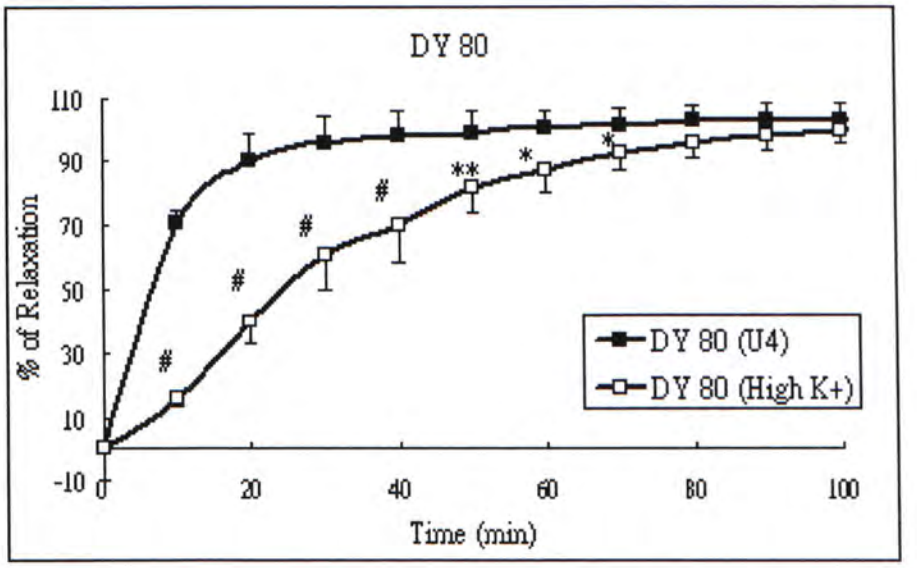


Fig. 4.6 Effect of High extracellular K^+ on DY 80-induced relaxation of intact aorta rings. *Precontraction was induced by U46619 and 60mM KCl krebs solution respectively before the addition of DY 80 (4mg/ml). Data were presented as mean ± SD (n= 5). * P<0.05, ** P<0.01, # P<0.001, DY 80 (U4) versus DY 80 (High K^+). The maximum inhibition was 55.16 ± 1.06%.*

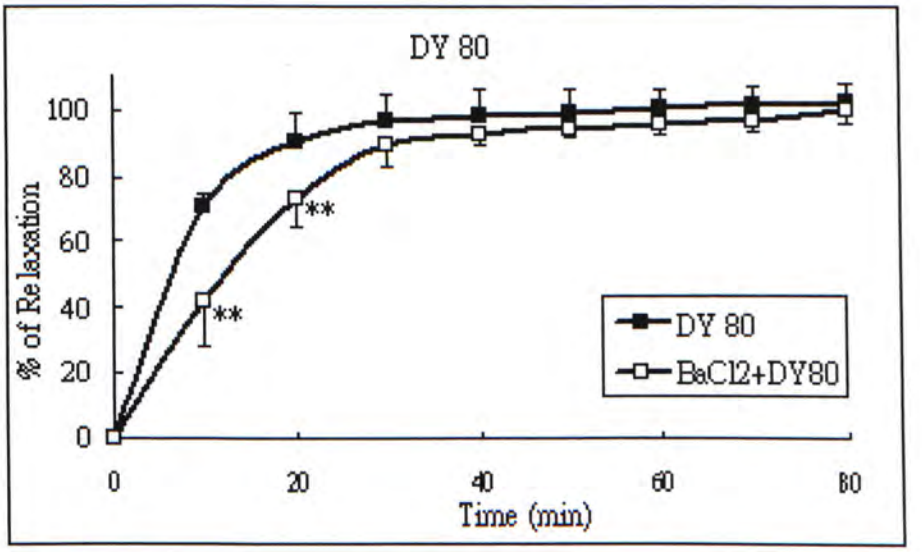


Fig. 4.7 Effect of Barium Chloride on DY 80-induced relaxation of intact aorta rings. *Barium Chloride (0.3mM) was added 30 minutes prior to the addition of DY 80 (4mg/ml). Data were presented as mean ± SD (n= 5), ** P<0.01. The maximum inhibition was 29.52 ± 9.58%.*

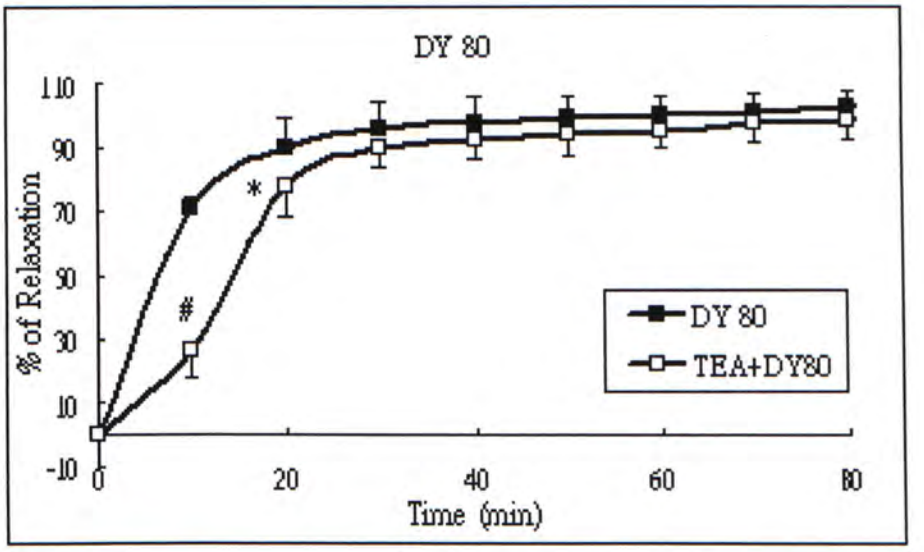


Fig. 4.8 Effect of TEA on DY 80-induced relaxation of intact aorta rings. *TEA (10mM) was added 30 minutes prior to the addition of DY 80 (4mg/ml). Data were presented as mean ± SD (n= 5), ** P<0.01, # P<0.001. The maximum inhibition was 44.37 ± 6.71%.*

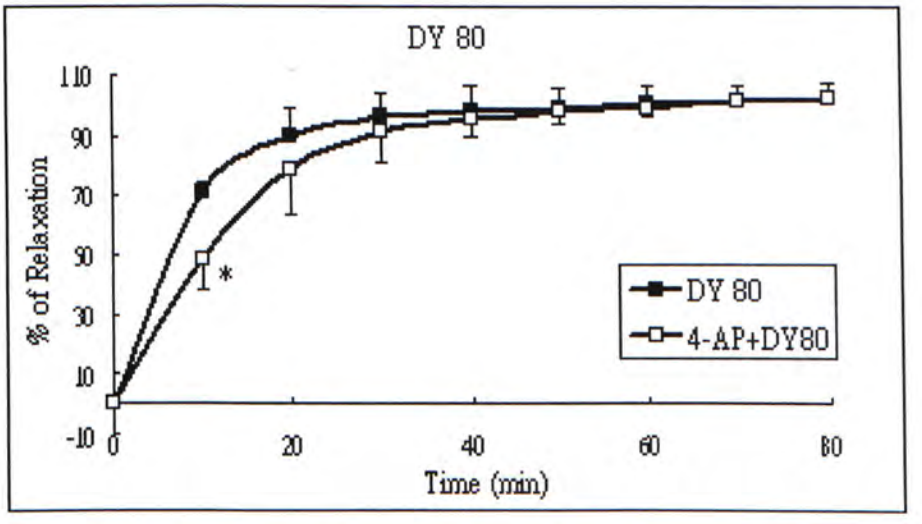


Fig. 4.9 Effect of 4-aminopyridine on DY 80-induced relaxation of intact aorta rings. *4-aminopyridine (3mM) was added 30 minutes prior to the addition of DY 80 (4mg/ml). Data were presented as mean ± SD (n= 5), * P<0.05. The maximum inhibition was 22.57 ± 8.05%.*

4.3.2 Salvianolic acid B

In figure 4.10 – 4.13, SAB showed no change in the relaxation response of aorta rings after the removal of endothelium and treatment with indomethacin. However, when rings were treated with L-NAME or ODQ, the relaxation response was partially but significantly inhibited. In figure 4.14, in the presence of high extracellular K^+, SAB-induced relaxation was completely abolished. Figure 4.15 - 4.17 showed that the treatment with $BaCl_2$ and TEA significantly inhibited the relaxation response of SAB and 4-aminopyridine could partially inhibit the effect.

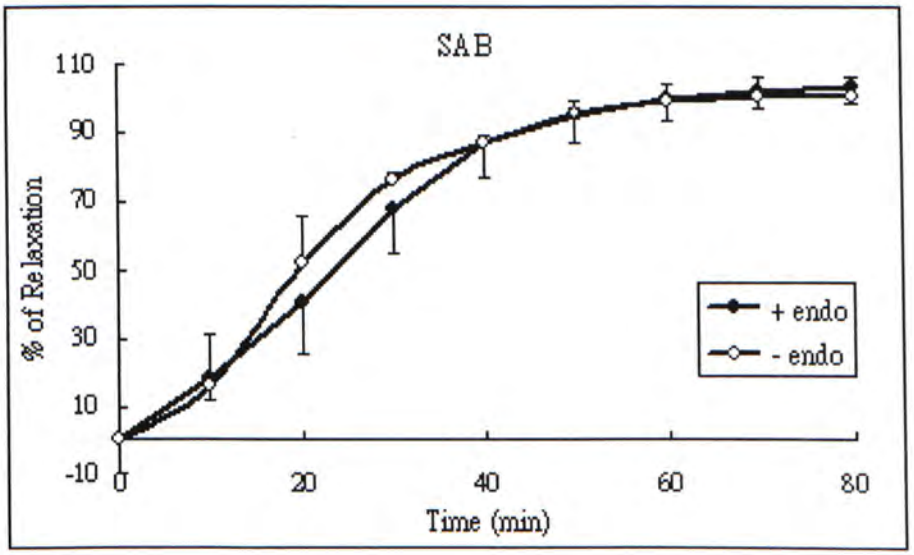


Fig. 4.10 Effect of denuded aorta rings on SAB-induce relaxation compared with intact rings. *Percentage of relaxation of the pre-contracted tone was compared in the course of time. "+ endo" represented the effect of SAB on intact aorta rings. "-endo" represented the effect on denuded rings. Data were presented as mean ± SD (n= 5). No inhibitory effect was shown.*

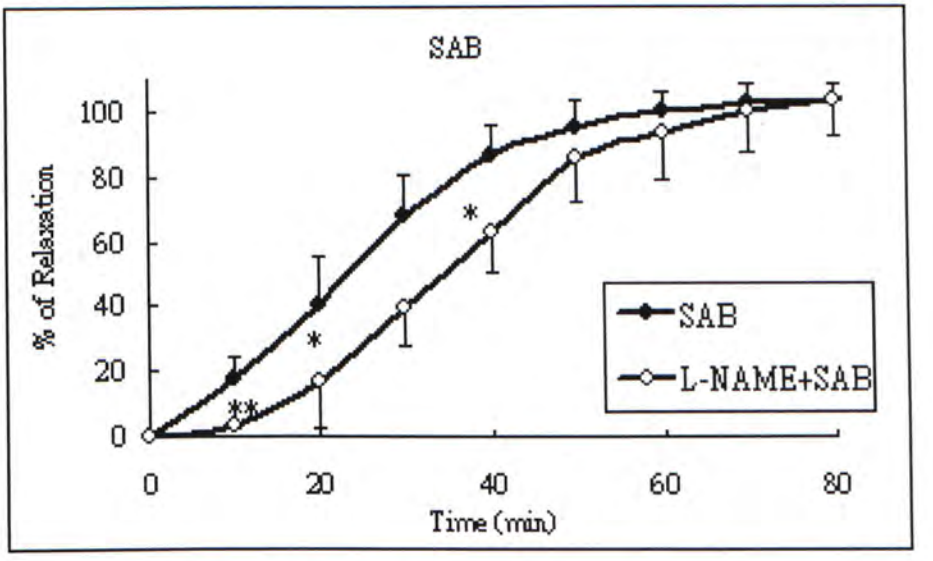


Fig. 4.11 Effect of L-NAME on SAB-induced relaxation of intact aorta rings. *L-NAME (100 μM) was added 30 minutes prior to the addition of SAB (0.245mg/ml). Data were presented as mean ± SD (n= 5), *P<0.05, ** P<0.01. The maximum inhibition was 28.47 ± 1.12%.*

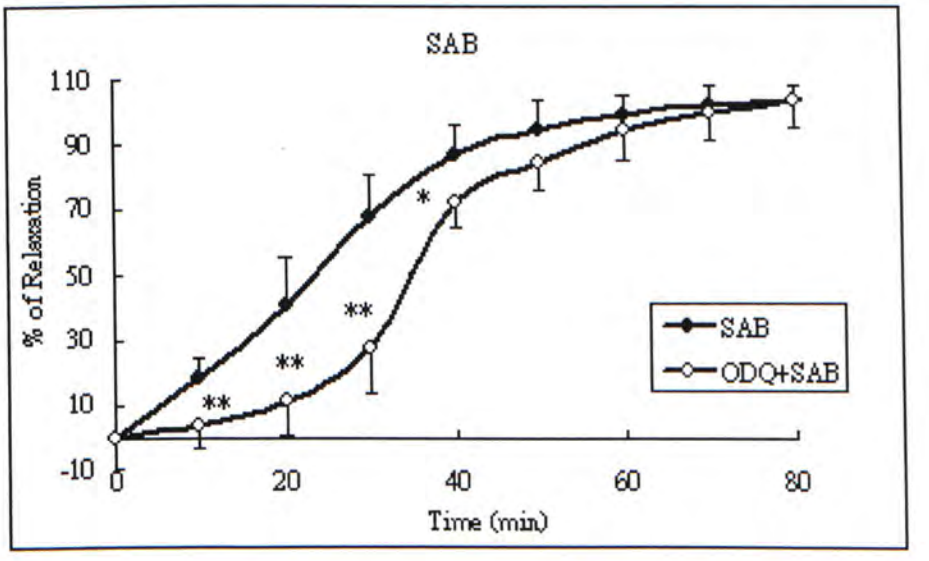


Fig. 4.12 Effect of ODQ on SAB-induced relaxation of intact aorta rings. *ODQ (3 μM) was added 30 minutes prior to the addition of SAB (0.245mg/ml). Data were presented as mean ± SD (n= 5), *P<0.05, ** P<0.01. The maximum inhibition was 40.43 ± 1.86%.*

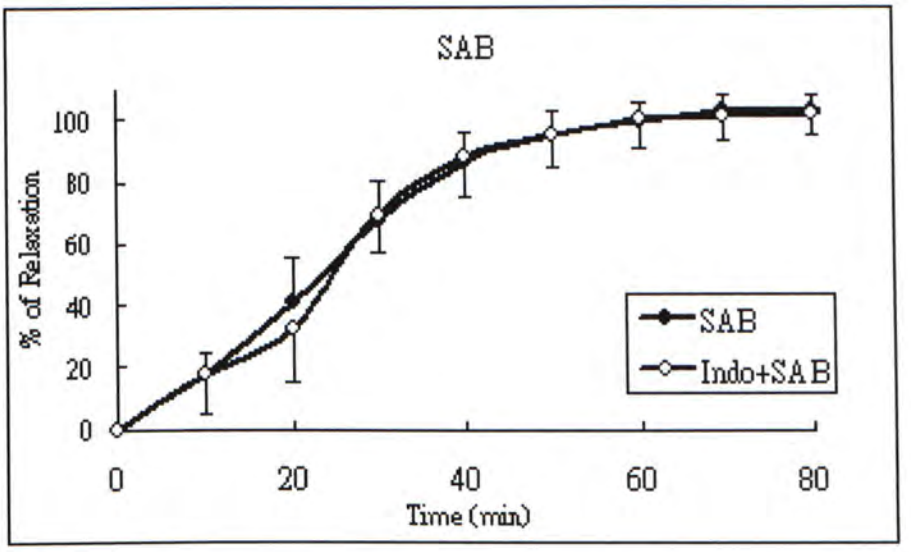


Fig. 4.13 Effect of Indomethacin on SAB-induced relaxation of intact aorta rings. *Indomethacin (1 μM) was added 30 minutes prior to the addition of SAB (0.245mg/ml). Data were presented as mean ± SD (n= 5). No inhibitory effect was shown.*

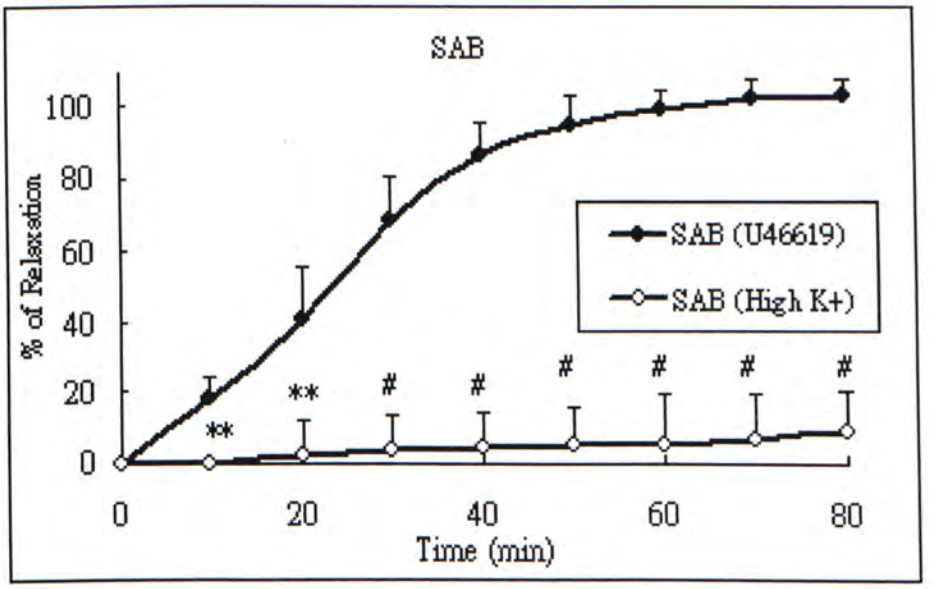


Fig. 4.14 Effect of High extracellular K^+ on SAB-induced relaxation of intact aorta rings. *Precontraction was induced by U46619 and 60mM KCl Krebs solution respectively before the addition of SAB (0.245mg/ml). Data were presented as mean ± SD (n= 4). ** P<0.01, # P<0.001, SAB (U4) versus SAB (High K^+). The maximum inhibition was 95.61 ± 5.21%.*

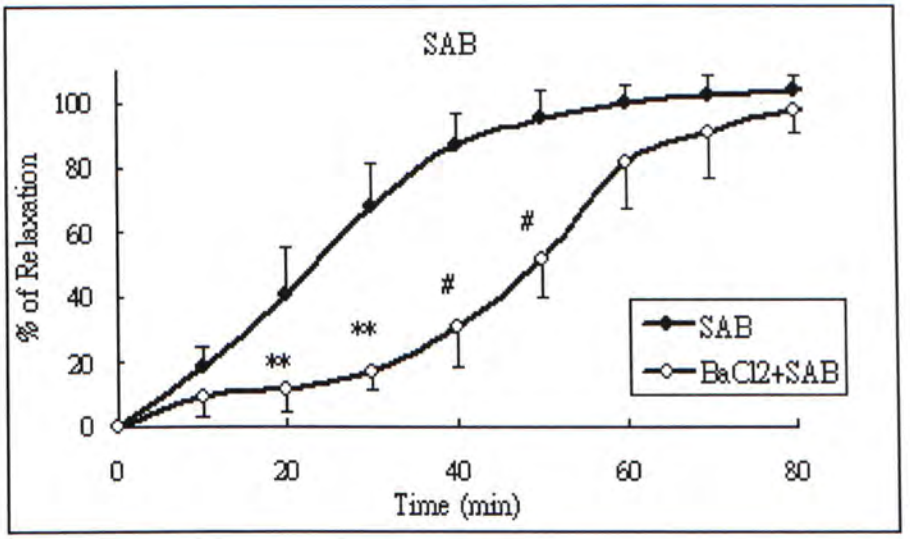


Fig. 4.15 Effect of Barium Chloride on SAB-induced relaxation of intact aorta rings. *Barium Chloride (0.3mM) was added 30 minutes prior to the addition of SAB (0.245mg/ml). Data were presented as mean ± SD (n= 5), ** P<0.01, #P<0.001. The maximum inhibition was 55.79 ± 4.83%.*

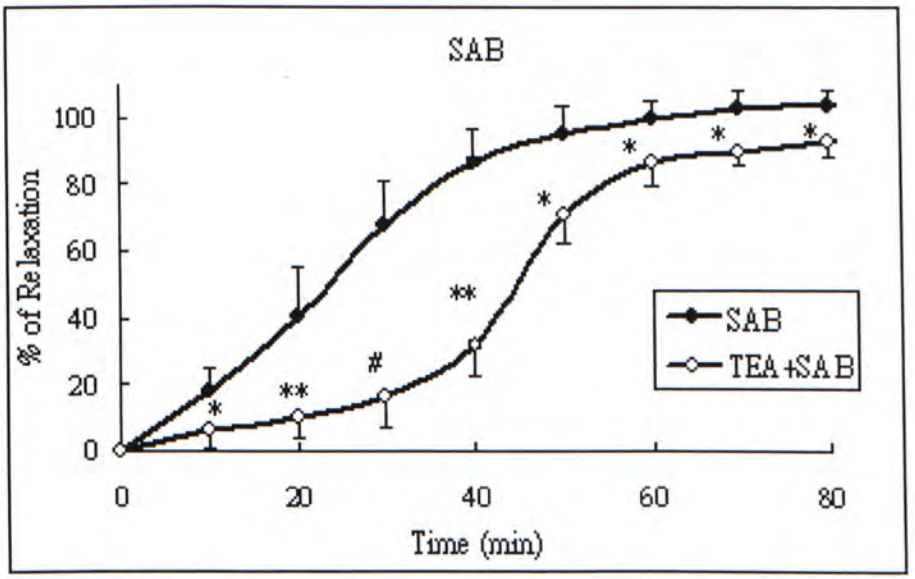


Fig. 4.16 Effect of TEA on SAB-induced relaxation of intact aorta rings. *TEA (10mM) was added 30 minutes prior to the addition of SAB (0.245mg/ml). Data were presented as mean ± SD (n= 5), *P<0.05, ** P<0.01, # P<0.001. The maximum inhibition was 54.7 ± 2.05%.*

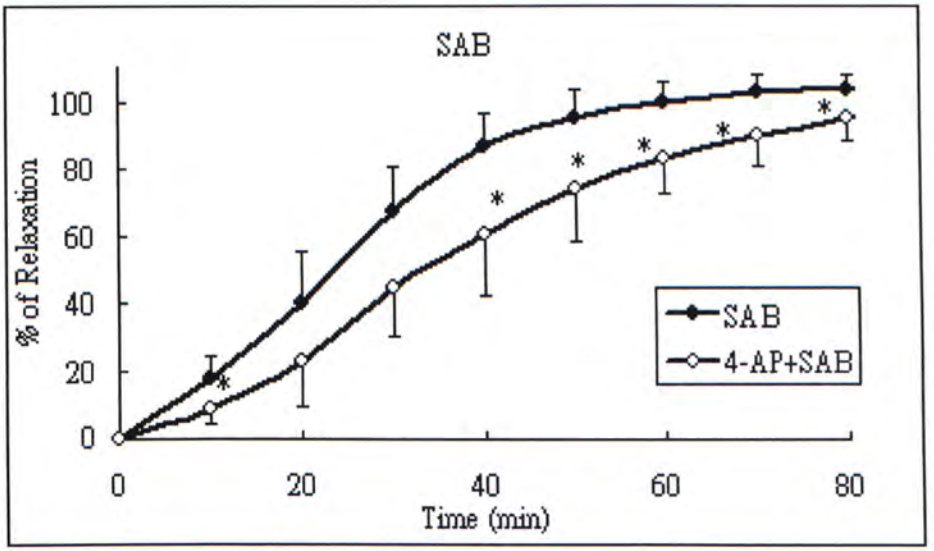


Fig. 4.17 Effect of 4-aminopyrdine on SAB-induced relaxation of intact aorta rings. *4-aminopyridine (3mM) was added 30 minutes prior to the addition of SAB (0.245mg/ml). Data were presented as mean ± SD (n= 5), * P<0.05. The maximum inhibition was 25.64 ± 10.08%.*

4.3.3 Daidzein

As shown in figure 4.18 – 4.21, the removal of endothelium layer and treatment with L-NAME, ODQ or indomethacin can significantly and effectively cause the attenuation of the relaxation response induced by daidzein. In figure 4.22, treatment with high extracellular K^+ completely abolished the daidzein-induced relaxation. Further examination showed that $BaCl_2$, TEA and 4-aminopyridine can cause almost complete abolition of the response induced by daidzein (figure 4.23 – 4.25).

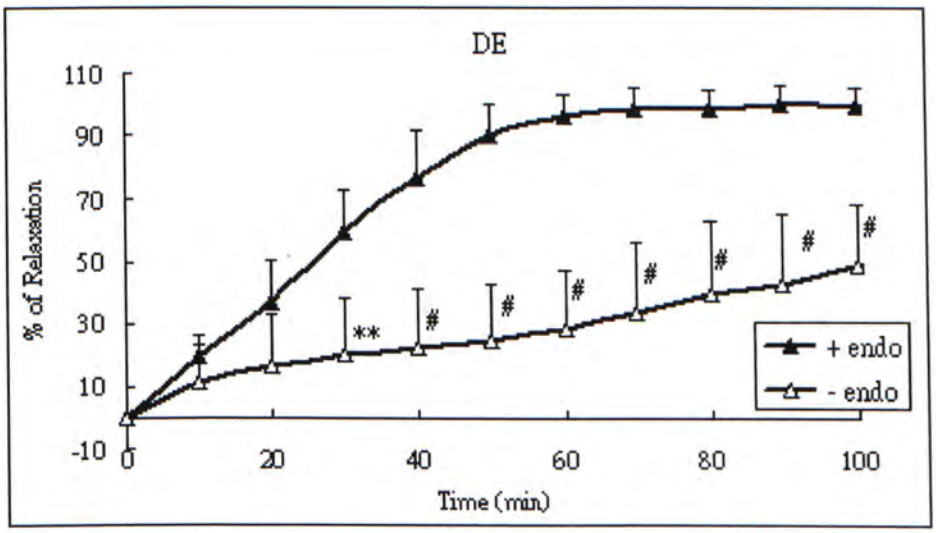


Fig. 4.18 Effect of denuded aorta rings on Daidzein-induce relaxation compared with intact rings. *Percentage of relaxation of the pre-contracted tone was compared in time course experiment. "+ endo" represents the effect of daidzein on intact aorta rings. "-endo" represents the effect of daidzein on denuded rings. Data were presented as mean ± SD (n= 6). The maximum inhibition was 68.16± 12.03%.*

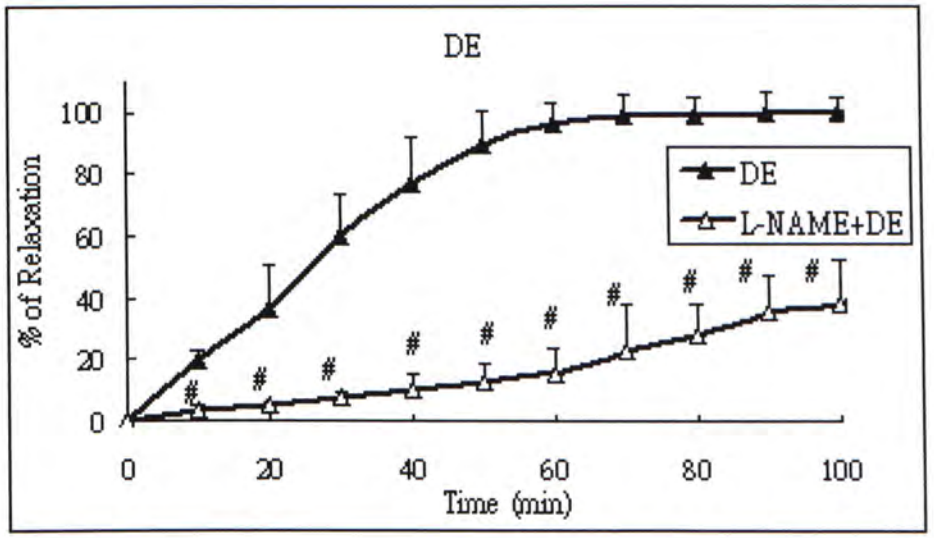


Fig. 4.19 Effect of L-NAME on Daidzein-induced relaxation of intact aorta rings. *L-NAME (100 μM) was added 30 minutes prior to the addition of daidzein (0.0056mg/ml). Data were presented as mean ± SD (n= 6), # P<0.001. The maximum inhibition was 80.54 ± 1.58%.*

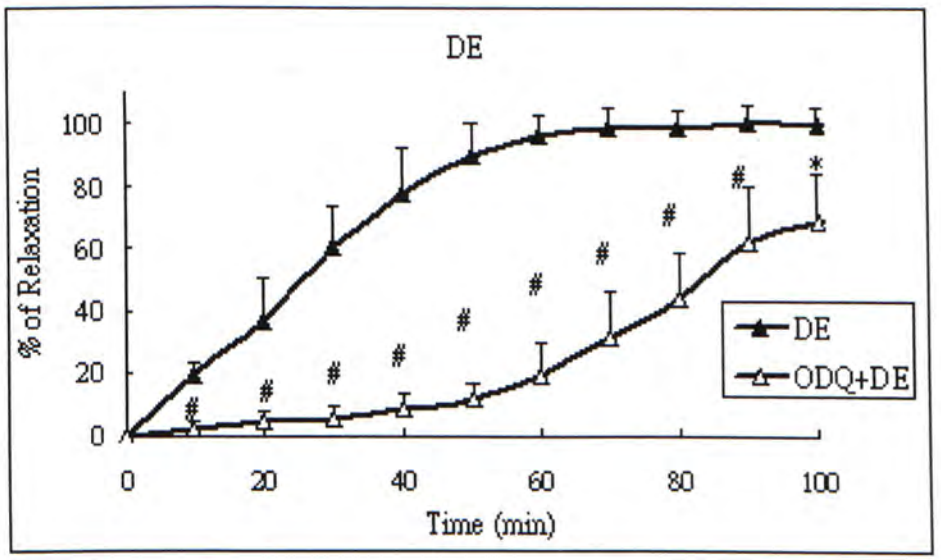


Fig. 4.20 Effect of ODQ on Daidzein-induced relaxation of intact aorta rings. *ODQ (3 μM) was added 30 minutes prior to the addition of daidzein (0.0056mg/ml). Data were presented as mean ± SD (n= 6), # P<0.001. The maximum inhibition was 79.41 ± 5.89%.*

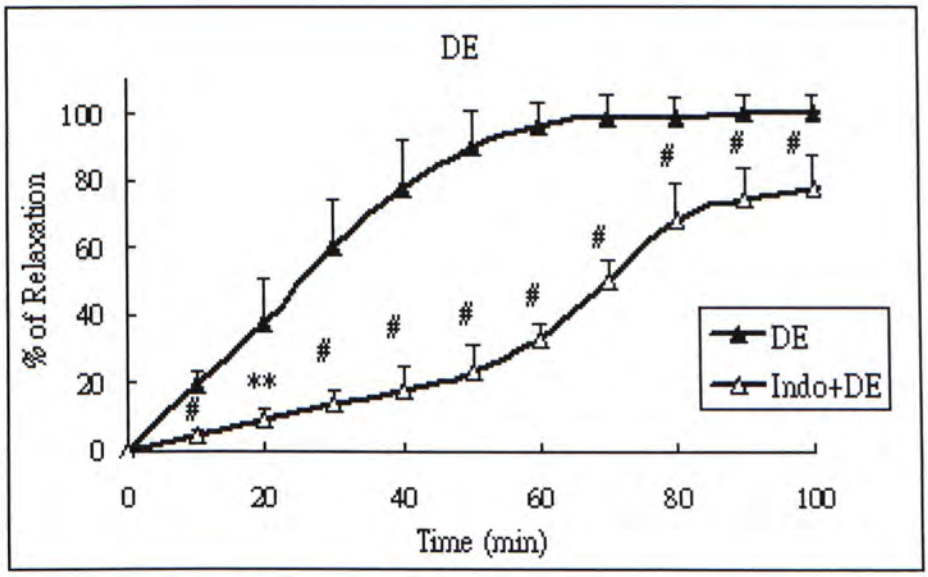


Fig. 4.21 Effect of Indomethacin on Daidzein-induced relaxation of intact aorta rings. *Indomethacin (1 μM) was added 30 minutes prior to the addition of daidzein (0.0056mg/ml). Data were presented as mean ± SD (n= 6), ** P<0.01, # P<0.001. The maximum inhibition was 67.33 ± 3.24%.*

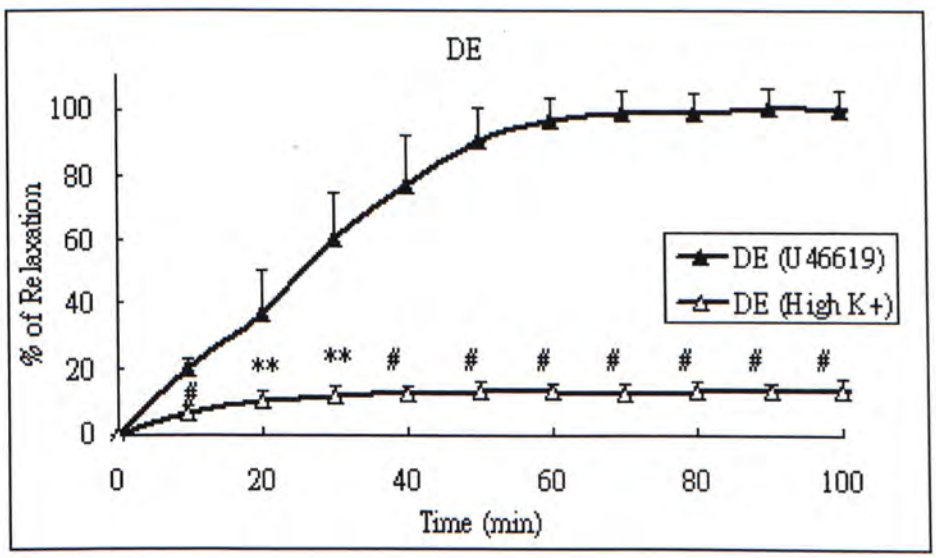


Fig. 4.22 Effect of High extracellular K^+ on Daidzein-induced relaxation of intact aorta rings. *Precontraction was induced by U46619 and 60mM KCl Krebs solution respectively before the addition of daidzein (0.0056mg/ml). Data were presented as mean ± SD (n= 5). ** P<0.01, # P<0.001, DE (U4) versus DE (High K^+). The maximum inhibition was 87.39 ± 2.83%.*

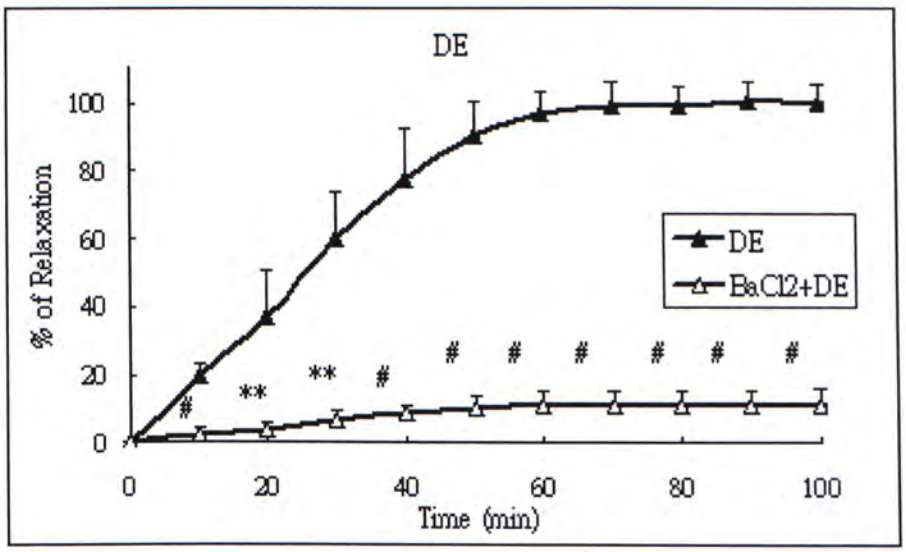


Fig. 4.23 Effect of Barium Chloride on Daidzein-induced relaxation of intact aorta rings. *Barium Chloride (0.3mM) was added 30 minutes prior to the addition of daidzein (0.0056mg/ml). Data were presented as mean ± SD (n= 4), **P<0.01, # P<0.001. The maximum inhibition was 87.54 ± 2.37%.*

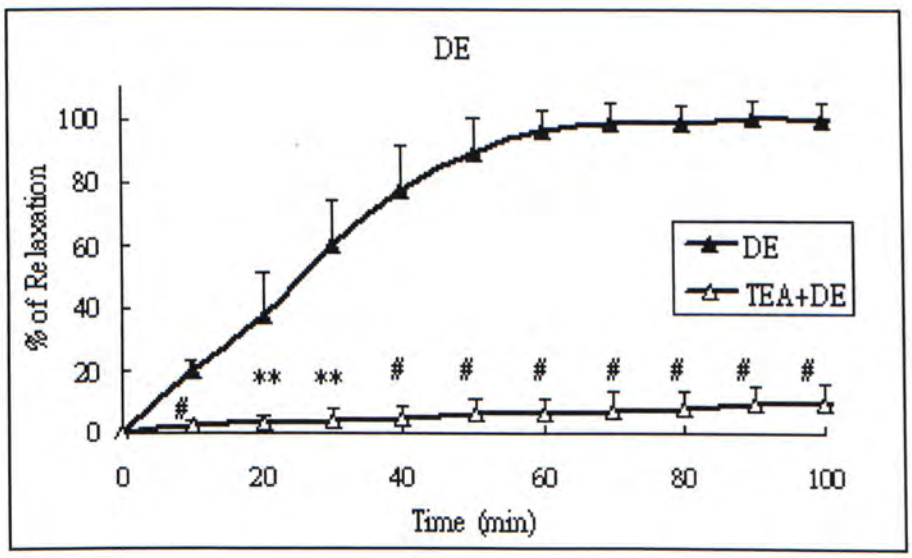


Fig. 4.24 Effect of TEA on Daidzein-induced relaxation of intact aorta rings. *TEA (10mM) was added 30 minutes prior to the addition of daidzein (0.0056mg/ml). Data were presented as mean ± SD (n= 5), **P<0.01, # P<0.001. The maximum inhibition was 90.8 ± 0.94%.*

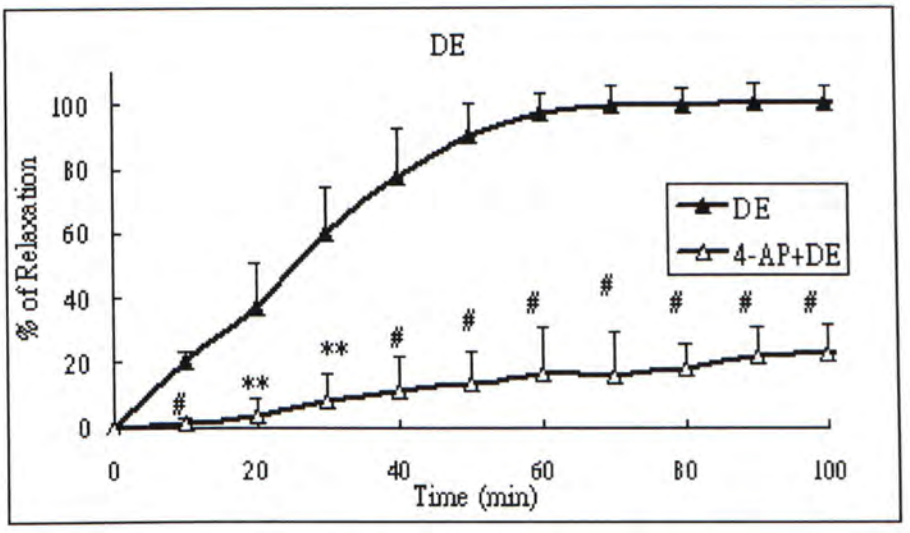


Fig. 4.25 Effect of 4-aminopyridine on Daidzein-induced relaxation of intact aorta rings. *4-aminopyridine (3mM) was added 30 minutes prior to the addition of daidzein (0.0056mg/ml). Data were presented as mean ± SD (n= 5), **P<0.01, # P<0.001. The maximum inhibition was 82.72 ± 7.14%.*

4.4 Discussion

In order to define the mechanism involved in the vasodilation response afforded by DY 80 extract and its active ingredients, the change in the relaxation profile were compared between aorta rings with intact endothelium and aorta rings with denuded endothelium, and the effects of specific blockers were examined. Should removal of endothelium abolish the relaxation effect of the drug, the drug may mediate an endothelium-dependent relaxation. Otherwise, an endothelium-independent relaxation could be inferred. L-NAME inhibits the enzyme nitric oxide synthase (NOS) which synthesizes nitric oxide (NO) in the endothelium. Released NO will activate the guanylate cyclase (GC)

in smooth muscle cell to initiate the relaxation, with the latter being inhibited by ODQ. Experiments using the two blockers aimed to examine whether the drug acts through the NO-mediated relaxation pathway. Besides, indomethacin inhibits the enzyme cyclooxygenase (COX), which synthesizes prostaglandin H2, the precursor of prostacyclin. This study aimed to examine whether the drug acts through the prostacyclin pathway. High extracellular K^+ generates vascular contraction response, if relaxation induced by a specific drug was abolished in reduced electrochemical gradient of K^+, the drug may act through the opening of potassium channels. $BaCl_2$ specifically blocks Kir channel, TEA is specific to BKca while 4-AP blocks the voltage-dependent Kv channel. Specific blockage of K^+ channel can define which channel is involved in the drug-induced relaxation response.

As illustrated in the earlier chapter, SAB and daidzein were the two major active compounds which contributed to the vasodilation activity of DY 80 extract. Results showed that action of SAB was either via the NO-dependent or EDHF-mediated relaxation pathways. Prostacyclin was not likely to be involved. Inhibition of the relaxation effect of SAB by treatment with L-NAME and ODQ proposed a NO-related relaxation mechanism. However, the effect of SAB was only partially abolished by the inhibition of NOS and

GC, implying that the action of SAB could also involve other pathways. High extracellular K^+ completely attenuated the relaxation response of SAB, which suggested the involvement of K^+ channel. Among the three types of K^+ channels investigated, SAB mainly acted on Kir and BKca, rather than Kv voltage-dependent channels. Interestingly, when the effect of SAB was examined in denuded aorta rings, it showed no difference in the vasodilating response compared to that of intact rings, indicating an endothelium-independent relaxation. Therefore, the main action of SAB is on the smooth muscle cells and the putative targets include guanylate cyclase and K^+ channels. The smooth muscle-mediated effects compensated the decreased availability of NO by removal of endothelial and maintained the relaxation tone of SAB in denuded rings. Therefore, the action of SAB can be concluded to be predominantly endothelium-independent and is mainly acting on the vascular smooth muscle.

Regarding daidzein, the intervention studies indicated that it acted through endothelium-dependent relaxation, presummably by the release of NO and prostacyclin. Daidzein also acted on the smooth muscle cells by opening the potassium channels since the blockage of the channels completely abolished its effect. Therefore, daidzein may act through both endothelium-dependent and

endothelium-independent smooth muscle cell mechanisms. Daidzein exhibited high efficacy in its action at low dosage. The mechanism underlying the non-selective but potent action of this pure compound is of much interest but remains to be confirmed by further studies, which may shed light on the prevention of atherosclerosis.

Referring DY 80 extract, it could be seen that inhibition in the NO and prostacyclin pathways did not change the vasodilatory response of DY 80. Therefore the endothelium seemed not to be the crucial factor for its effect. DY 80-mediated vasodilation was found to be inhibited by the blockage of potassium channels, albeit to a partial extent. Therefore, it could be inferred that SAB is the active compound which contributed more to the DY 80's effect, because of its higher content in the extract when compared with daidzein. In the absence of endothelium, the effect of SAB and daidzein on smooth muscle cells were able to account for the relaxation response of DY 80. However, it is also possible that other compounds could be the active components of DY 80, apart from SAB and daidzein. As vasodilation is a complex process, these compounds, together with SAB and daidzein, may also act through other mechanisms in achieving the relaxation. Other factors, which may mediate endothelium-independent relaxation, include the direct inhibitory action on the

influx of Ca^{2+} into the smooth muscle cells and the release of Ca^{2+} from the intracellular stores, inhibition of cyclic nucleotide phosphodiesterase and inhibition of protein kinase such as myosin light chain kinase and other kinases involved in Ca^{2+}-sensitizing such as protein kinase C, Na^{+} -K^{+}- ATPase and cytochrome 450. However, the following questions remain unsolved in the present study. How could daidzein achieve its multi-target actions? What makes SAB possible to induce the vasodilation response with potency similar to that of daidzein, by predominantly acting on potassium channels only? And what will be the explanation for the observation that the action mechanisms of daidzein did not reflect in that of the crude extract DY 80? Further in-depth study is needed to obtain more understanding about the working mechanism of DY 80 and its active compounds on vasodilation, which is enabled by the treatment of inhibitors/blockers in combinations, to reconfirm their involvement in specific relaxation pathways (NO, PGI_2, EDHF, etc).

Vasodilation property provides a surrogate marker to assess the endothelial function of vessels. Normal endothelium embraces anticoagulant, anti-platelet and fibrinolytic properties, as well as controls vascular tone and smooth muscle cell growth. Endothelial dysfunction contributed to disease states characterized by vasoconstriction, excessive thrombosis, abnormal vascular proliferation,

hypertension and atherosclerosis. These abnormal endothelial physiologies were implicated in both early-stage and advanced-stage atherosclerosis, which may be related mainly to the decreased bioavailability of nitric oxide (NO). Studies reported that the decreased local availability of NO or excess production of superoxide anions was accompanied by endothelial dysfunction. NO is a local vasodilator and it can inhibit platelet adherence and aggregation, smooth muscle proliferation and leucocytes interaction with endothelium. Reduced NO can result in proatherogenic effects even at gene transcription level, contributing to the initiation and progression of atherosclerosis. In the present mechanistic study, a putative NO-mediated relaxation pathway was demonstrated to account for vasodilation effect of daidzein and partially for that of SAB. These two active compounds therefore might be able to reverse the condition of endothelial dysfunction in patients by restoring NO, providing possibility to deter the progression of atherosclerosis. Prostacyclin also plays an important role in inhibiting platelet adhesion to endothelial cells. Daidzein was also shown possible to increase availability of prostacyclin. All these actions might help reversing endothelial dysfunction and promote a healthier vessel. It was also reported that impaired endothelium-dependent dilation at sites of coronary plaques may result in paradoxic vasoconstriction during exercise or mental stress, when vasodilation is mostly needed. Vasodilating

effect of these two active compounds may alleviate these high risk acute conditions. If these strategies could be implemented early in the disease process, it might prevent or retard atherogenesis. Even in advanced stage, improvement of vascular health may help decrease chances of thrombosis and vasoconstriction, hence decrease the risk of acute cardiovascular events.

Conclusion

To conclude, the vasodilation effect of DY 80 extract was endothelium-independent and partially acts on opening of K^+ channels. SAB acted through endothelium-independent relaxation and on smooth muscle K^+ channels. Daidzein acts via endothelium-dependent vasodilation and non-selectively through nitric oxide, prostacyclin and EDHF pathways. These two active compounds and the crude extract were found possible to increase bioavailability of NO and decrease free radical production by their antioxidant property, as previously mentioned, may help reverse endothelial dysfunction and slower the progression of atherosclerosis.

Chapter 5

Study on Lipid Peroxidation and Uptake by Macrophages

5.1 Study of DY 80 and SAB on Copper-ion induced Low Density Lipoprotein Oxidation

5.1.1 Pathologic Role of oxidized Low Density Lipoprotein

Oxidized LDL (oxLDL) plays an important role in the pathogenesis of atherosclerosis, both in the early inflammatory stage (Steinberg, 1997) and the advanced (Auge et al., 1999) stage of atherosclerotic lesion. Oxidized LDL induces foam cells transformation. Besides, oxidation of LDL in subendothelial space converts LDL into ligands of the scavenger receptors and the receptor-mediated endocytosis of oxLDL may be a primary pathway for the cholesterol deposition in macrophages during plaque formation *in vivo*. OxLDL is cytotoxic to endothelial cells and is chemotactic to monocytes, increasing their extra-cellular matrix synthesis and promotes platelet aggregation (Parthasarathy & Rankin, 1992).

5.1.2 Antioxidants in Low Density Lipoprotein and Role of Transition Metals

The main lipophilic antioxidant inside LDL is vitamin E (α -tocopherol) which resist ox-LDL formation (Esterbauer et al., 1992) and impair the leukocytes superoxide production (Cachia et al., 1998). Any exogenous source of antioxidants, which could act together to further resist or delay oxidation of these lipoproteins, are considered beneficial in preventing lipid peroxidation and relieve atherosclerosis. Lipid peroxidation was induced by Cu^{2+} *in vitro* in the present study. The presence of transition (e.g. Cu^{2+}, Fe^{2+}) metals in atherosclerotic lesions has been reported (Lamb et al.., 1995; Stadler et al., 2004) which may reveal the possible role of transition metals in lipid peroxidation. DY 80 extract and SAB were examined in this study for their potency to resist Cu^{2+}-induced lipid peroxidation.

5.1.3 Methods and Materials

Preparation of LDL

Human LDL (Merck, 10mg) was washed by passing through HiTrapTM Desalting column (Amersham Pharmacia Biotech, 5ml) to remove EDTA. PBS was then used to elute the native LDL.

LDL Oxidation

Oxidation was initiated by the addition of $CuSO_4$ (final Cu^{2+} concentration 5uM) to the LDL (final concentration 100ug/ml). The formation of conjugated dienes was monitored continuously by measuring the increase in absorbance at 234 nm. The LDL suspension was incubated in a spectrophotometer cuvette at 37°C for 24 hours. The spectrophotometer was set to zero against the blank and the increase in the absorbance during LDL oxidation was recorded every 8 minutes. The lag time required for the initiation of lipoprotein oxidation was determined from the oxidation curve.

Inhibition of lipid peroxidation by drugs

Appropriate concentrations of drugs were added to the native LDL before the addition of oxidation-initiating agent Cu^{2+} into cuvettes. Any change in the lag time for the formation of conjugated dienes in the presence of drugs was compared with the control.

5.1.4 Results

Lag time determination was defined by the end of lag phase which is accompanied by the start of propagation phase, as described (figure 5.1). As shown in figure 5.2 and 5.3, DY 80 and SAB were found to be able to delay the LDL oxidation by shifting the oxidation curve to the right, compared with the control without drug. In addition, by doubling the concentration of DY 80 and SAB, the lag times of dienes formation were further delayed by two fold, showing a dose-dependent effect.

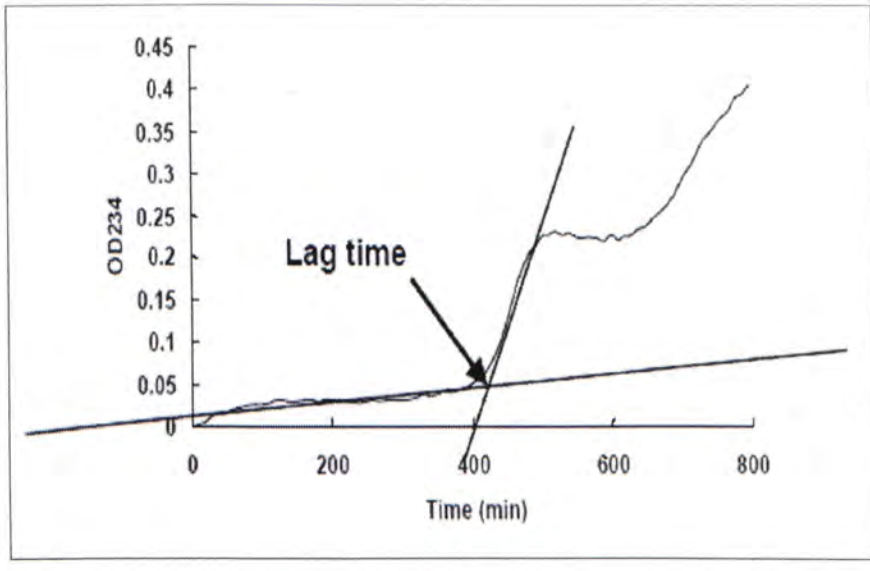


Fig. 5.1 Determination of lag time in Cu^{2+}-induced LDL oxidation model. *Formation of conjugated dienes was monitored for 24 hours at 234nm wavelength. The lag time is defined as the intercept point of the two crossed lines.*

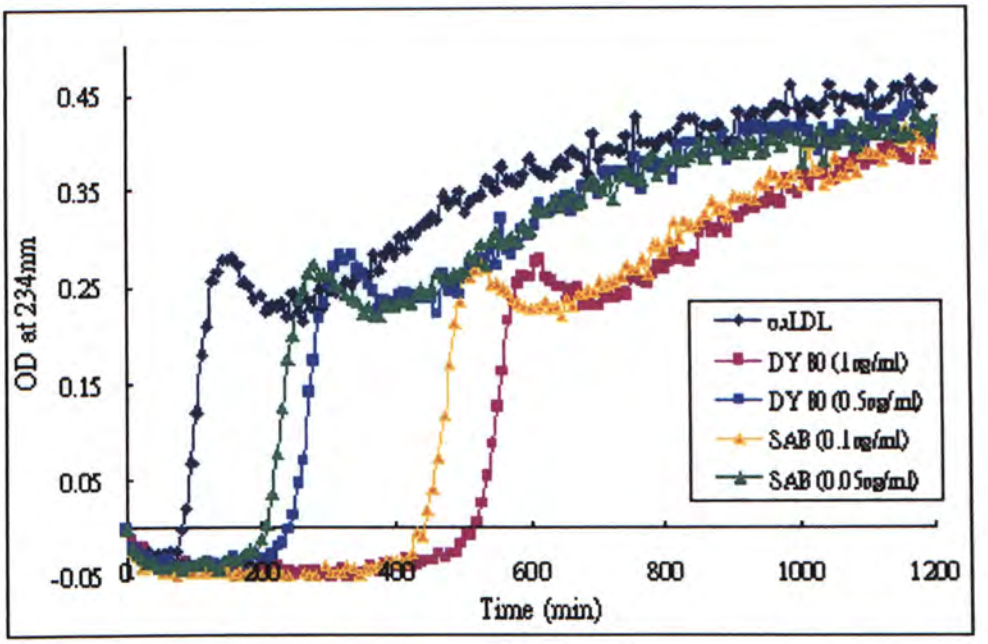


Fig. 5.2 Antioxidative effect of DY 80 and SAB on Cu^{2+}-induced LDL oxidation. *Data of one of seven trials was shown here. The lag time for the trial was determined as oxLDL (control) 128min, DY 80 (1μg/ml) 632min, DY 80 (0.5μg/ml) 320min, SAB (0.1μg/ml) 536min and SAB (0.05μg/ml) 288min.*

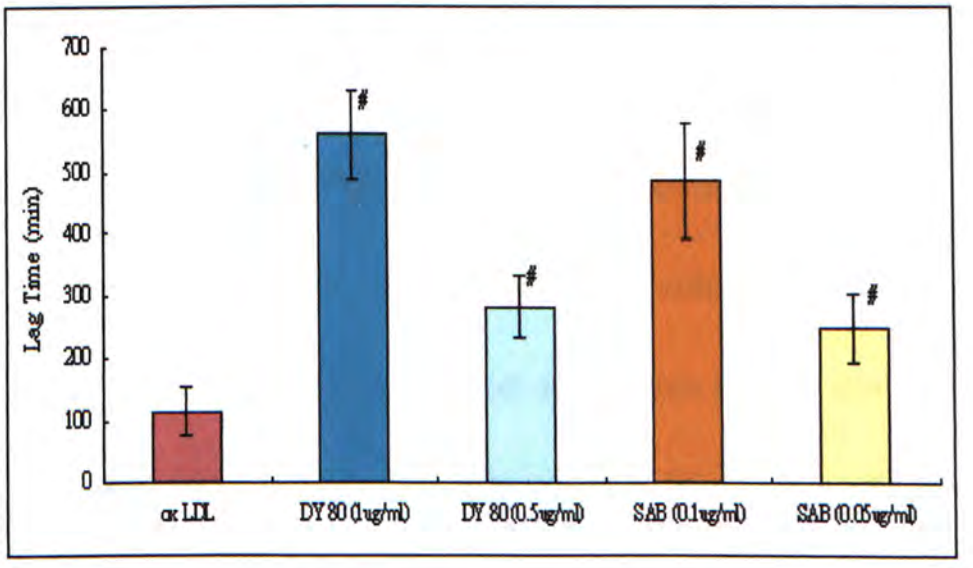


Fig. 5.3 Deferral of Lag Time for coupled diene formation by DY 80 and SAB on Cu^{2+}-induced LDL oxidation. *Average lag time for coupled dienes formation with drugs or without drug were presented. Data were expressed as mean ± SD (n=7). # P<0.001, compared with control. Both concentrations of DY 80 and SAB were found able to delay the lag time for dienes formation.*

5.1.5 Discussion

Free radicals are generated elsewhere in the body and can cause lipid peroxidation. For example, superoxide anions are generated from xanthine/xanthine oxidase in the endothelial cells. Mitochondria are also an important source of oxygen free radicals. Free radicals are also formed in leukocytes, and during the oxidation of catecholamines. Oxidized LDL is capable of a wide range of toxic effects. OxLDL activates inflammatory processes at the level of gene transcription by up-regulation of NF-κB, expression of adhesion molecules, and recruitment of leukocytes, hence increases endothelial cell adhesiveness towards monocytes (Lamb et al., 1995; Steinbrecher et al., 1990). It also promotes vascular smooth muscle cells proliferation (Auge et al., 1999a). OxLDL can inhibit the production of nitric oxide (NO), and induces vessel wall dysfunctions that are characteristically associated with development of atherosclerosis. Therefore, resisting oxidation of lipoproteins may lead to the possibility of relieving endothelial dysfunction by reducing loss of NO.

SAB has been identified previously as antioxidants, belonging to the group of polyphenols. Polyphenols are known to be antioxidant and protect other antioxidants in the biological fluids and LDL *in vivo* (Carbonneau et al., 1997; Cartron et al., 2001). Drug treatment with either DY 80 extract or SAB both significantly reduce the rate of lipid peroxidation in this *in vitro* study, which may further suggest their putative anti-atherogenic effect in the prevention of cardiovascular disease and add values to this herbal formula, as preventing LDL oxidation is a critical step in deterring atherosclerosis, especially at early stages.

5.2 Study of Scavenger Receptor Regulation in Macrophages

5.2.1 Introduction

Peroxidation of low density lipoprotein and subsequent excessive uptake of the oxidatively-modified LDL via the scavenger receptor into macrophages are believed to play an important role in atherogenesis (Esterbauer et al., 1997). It is recognized that several types of scavenger receptor proteins are expressed on the macrophage surface, including SR-A type I, II and III, MARCO, CD36, SR-BI, CD68, LOX-1, PSR and SR-PSOX (Kodama et al., 1990; Greaves et al., 1998; Ottnad et al., 1995). SR-A type I and type II are the first identified members of the scavenger receptor family (Rohrer et al., 1990; Kodama et al., 1990). Several studies have documented important roles of SR-A in atherosclerosis. Marked reduction in atherosclerosis was demonstrated in mice lacking the gene encoding SR-A (Babaev et al., 2000). Macrophages lacking both SR-A and CD36 have 80 - 90% reduction in the internalization and degradation of either acetylated or oxidized lipoproteins (Kunjathoor et al., 2002). Therefore, the effect of DY 80 and SAB on the SR-A receptor expression was evaluated in this study.

5.2.2 Methods and Materials

THP-1 cell line

Human peripheral blood acute monocytic leukemia cell line THP-1 was obtained from American Type Culture Collection (ATCC, Roctvile, MD, USA). Cells were grown in suspension at 37°C in 5% CO_2, 95% air in RPMI 1640 medium containing 10% fetal bovine serum (FBS) (v/v) (GIBCO-BRL). Cells were plated and differentiation was induced by 60nM PMA co-incubation for 72 hours before used in experiments. The cells were then incubated with or without lipoproteins (100ug/ml) and drugs.

Oxidized LDL and drugs preparation/treatment

Oxidized LDL was prepared by incubating freshly prepared LDL (600μg/ml) as mentioned in previous section, with 30μM $CuSO_4$ at 37°C in dark for 24 hour. DY 80 extract and SAB were suspended in the medium at appropriate concentrations. Both oxidized LDL and drugs were micro-filtered to remove contaminants before co-incubation with THP-1 cells. Final concentration of oxidized LDL in plate was 100μg/ml with DY 80 or SAB. The plates were kept in 5% CO_2, 37°C incubator for 48 hours before protein extraction.

Protein preparation

After drug treatment, the medium was discarded. Cells were washed and trypsinized, collected by centrifugation at 1000 x g for 3 minutes. Cell pellet was then washed with PBS before lysing in 30-50μl lysis buffer (1mM Phenylmethylsulfonylfluoride, 1% Triton X-100, 21 μg/ml Aprotinin, 0.5 μg/ml Leupeptin, 4.9 mM $MgCl_2$ and 1 mM Vanadate) on ice for 2 hours with frequent mixing to ensure complete lysis reaction. Cell lysates were then boiled at 100°C for 10 minutes. After centrifugation, supernatant was collected and stored at -70 ° C until use. Protein concentrations were determined by bicinchonic acid (BCA) assay (Sigma, USA).

Western Blotting

Equal amounts of proteins (30μg) were separated by 12.5% SDS-PAGE and transferred onto nitrocellulose membrane using Trans-Blot apparatus (Bio-Rad). After blocking with 10% non-fat milk for 1 hour at room temperature and washed three times TBS-T, each for 15 minutes, the membrane was incubated with primary antibody (Anti-SR-A, Chemicon International, Temecula, CA) with 1:1000 dilution at room temperature for 1 hour. The membrane was then washed three times with TBS-T, each for 15 minutes and incubated with anti-goat secondary antibodies which conjugated

with horse-radish peroxidase at 1:1000 dilution (Santa Cruz, USA) at room temperature for 1 hour. After washing, the membrane was visualized by chemiluminescence reagent of ECL system (Amersham Life Science, UK) and then exposed to X-ray film. The protein bands were normalized to unit actin (stained by anti-β-actin antibody (Sigma, USA) with 1:5000 dilution in primary antibody blotting and 1:1000 dilution in anti-mouse secondary antibody).

5.2.3 Results

Study of SR-A scavenger receptor protein expression on macrophages was presented in figure 5.4. Up-regulation of SR-A expression was found to be induced by oxidatively modified LDL. When macrophages and oxidized LDL were co-incubated with DY80 extract or SAB, they showed a significant down-regulation of the SR-A protein expression on macrophages. Two different concentrations of DY 80 and SAB were examined, however, they did not show obvious dose-dependent effects.

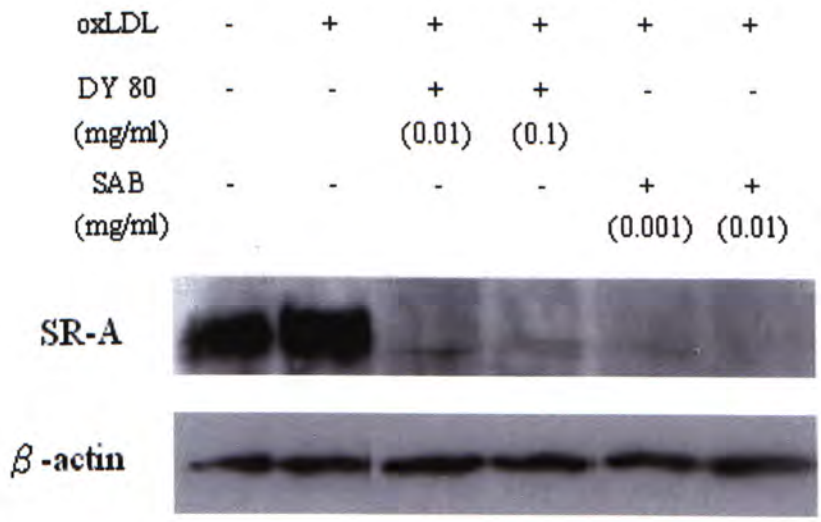


Fig 5.4 Western Blot of Scavenger Receptor A expression on THP-1 derived macrophage. *Anti-SR-A antibody was used to probe the SR-A protein (molecular size: 75kDa) expression in cell lysates. "+" and "-" represented the presence or absence of specific agents respectively. 100μg/ml oxidized LDL was co-incubated with drugs, DY 80 and SAB at different concentrations. MTT cell viability assay did not show toxicity effect by oxidized LDL or by DY 80 and SAB to cells for two day-incubation. β-actin was used for normalization.*

5.2.4 Discussions

Normal macrophages express very few LDL receptors of classical type. And it was found that LDL after modification, loses its ability to bind to classical receptors. They are instead, recognized by another high-affinity receptor, the scavenger receptors, which bind oxidized and acetylated LDL and uptake into macrophages (Brown & Goldstein, 1983). Scavenger Receptor Type A (SR-A) was found to be the most important class. SR-A gene knockout mice bred either on atherosclerosis-susceptible apolipoprotein E (apoE) knockout mice or LDL receptor knockout mice showed marked reduction in the size of atherosclerotic lesions (Sakaguchi et al., 1998; Suzuki et al., 1997).

In the present study, at both tested concentrations of DY 80 and SAB, marked suppression of the SR-A expression was observed, with no obvious dose response being exhibited. This may be due to the fact that the concentrations used in this test were too high for generating a significant dose-dependent effect. The effect of lower concentrations of the drugs should be further studied. The result of the present study suggested that DY 80 extract and active compound SAB, which have shown inhibitory activity on LDL oxidation, might be able to suppress also the scavenger receptor expression on macrophage surfaces, and hence reduce oxidized LDL uptake into

macrophages and inhibit foam cells formation.

Nevertheless, originally detected on macrophages (Gowen et al., 2000), SR-A is also present on other cell types, including smooth muscle cells and endothelial cells (Bickel et al., 1997). SR-A was found to have other biological properties that could impact the atherosclerotic process. One of these recently defined properties is its adhesion to lymphocytes (Yokota et al., 1998). The adhesive property may be particularly prominent in regions of damaged extracellular matrix (Gowen et al., 2000), as this would be expected to occur during inflammatory processes within atherosclerotic lesions. Apart from the pathology involved by SR-A expression, it was found that the total uptake and accumulation of LDL in macrophages may also be achieved via non-receptor-mediated pathway by which foam cells could form *in vivo* (Moore et al., 2005; Kruth et al., 2005). Previous studies have shown that enzyme modifications by exposing LDL to secretory phospholipase A2 can convert native LDL into a structure that can readily trigger foam cell formation and hence facilitate atherosclerosis (Zalewski & Macphee, 2005). On the other hand, study has showed that the role of SR-A deficiency in atherogenesis may be complicated by the presence or absence of apoE in very low density lipoproteins (vLDL) *in vivo* (de Winther et al., 1999). Overall the molecular

mechanism by which SR-A function is regulated remains unclear. Therefore, although our drugs could suppress SR-A expression on macrophage *in vitro*, the overall effect of DY 80 and SAB on the oxidized LDL uptake *in vivo* remains to be further elucidated.

Chapter 6

General Discussion

Cardiovascular disease is a prevalent disease in the century and is complicated by multiple factors. These factors are inter-connected that they accelerate the process of atherogenesis. Among the few properties investigated in this project, experimental evidence showed that the deficiency of one factor may affect the others. Reduced availability of NO in endothelial dysfunction contributes to the pathogenesis of atherosclerosis, and this reduction of NO can be mediated by oxidized LDL, or excessively consumed by superoxide radicals and other sources of reactive oxygen species. Oxidized LDL also reduces endothelial NO release through its effects on eNOS activity and gene expression, and scavenges NO or EDHF directly (Blair et al., 1999; Chin et al., 1992). Therefore, the oxidative stress and decreased bioavailbility of NO are closely related in accerlerating the progression of atherosclerosis.

In the present study, the 80% ethanol extract of Danshen-Yege formula and salvianolic acid B, being an active compound isolated from Danshen, showed potent antioxidant activity. The antioxidant action can protect low density lipoprotein from oxidation and thus inhibit the progression of atherosclerosis.

At the same time, daidzein, being an active compound present in Yege, showed vasodilation property in isolated aortic rings, presumably through the enhancement of NO release, which propose its possibility of restoring NO level *in vivo*. DY 80 and SAB, though act mainly via hyperpolarizing smooth muscle cells, may contribute to the reduction of severe hypertension in NO pathway-impaired patients. These multiple effects of Danshen-Yege herbal extract may be beneficial in preventing the development of atherosclerosis.

In addition to the present study, studies from our laboratory has demonstrated the cardiotonic effects of the Danshen-Fenge water extract, showing anti-proliferative effect against PDGH-induced proliferation and anti-migratory effect against platelet-derived growth factor-induced migration vascular smooth muscle cells *in vitro* model and anti-atherosclerotic effect *in vivo*, by exhibiting hypocholesterolemic effect on diet-induced hyperlipidemia in rabbit model and inhibiting the atheroma formation (Koon, 2006). A study from our Australian collaborates also showed that the formula could decrease foam cell formation by modulating acetylated LDL uptake, but in contrast to most studies, the extract increased the level of adhesion molecules *in vitro* (Sieveking et al., 2005). Furthermore, a clinical study indicated that the treatment with Danshen-Fenge water extract could improve several

atherosclerotic markers, including the lipid profile (total cholesterol and LDL-cholesterol amount) and vascular functions (flow-mediated brachial artery endothelium-dependent dilatation, nitroglycerin-induced dilatation and carotid intima-media thickness), in patients suffering from coronary heart disease (Tam, 2004). The homocysteine and adhesion molecules profiles of the patients were also monitored, however, no significant change was observed in the treatment group patients compared with placebo group.

Although pharmacological studies have suggested the therapeutical application of Dashen-Gegen formula in the prevention/treatment of atherosclerosis, the clinical efficacy has yet to be confirmed. Besides, the use of ethanol in the extraction process and Yege species in the formulation were found to produce a better pharmacological profile in preventing atheroslcerosis. However, further studies are needed to assess its effect *in vivo* and possibly in clinical trials in the future. Apart from the active ingredients, whether the ethanol extraction also yields more toxic products from the herbs at the same time remains to be determined. Furthermore, although the 80% ethanol extract showed good anti-atherogenic properties, whether this can be translated into clinical improvement remained to be tested by large scale clinical trials. Lastly, the development of atherosclerosis begins in youthful age, and progress rapidly

in prevalence during 15-34 of age (Strong et al., 1999). Therefore, although clinical symptoms of atherosclerosis are not usually manifested until later years of adulthood, primary prevention should begin in early age. As such, the use of Chinese herbal medicine, like Danshen-Gegen formula, may be warranted.

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